THE FATE OF FLUORESCENT WHITENING AGENTS IN THE AQUATIC ENVIRONMENT

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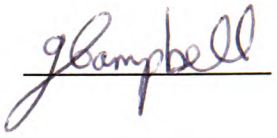
The Thames Water Authority

DECLARATION

This thesis has not been nor is currently submitted for the award of any other degree or similar qualification.



J CAMPBELL

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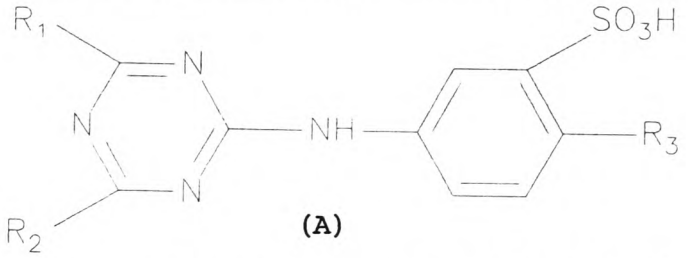
ABSTRACT

THE FATE OF FLUORESCENT WHITENING AGENTS IN THE AQUATIC ENVIRONMENT. J CAMPBELL

The commercial use of fluorescent whitening agents (FWA's) in products such as soaps and detergents has resulted in the presence of these compounds in domestic waste waters.
A model compound (A) (a possible initial degradation product of a stilbene-s-triazine type FWA) was synthesised and biodegradation studies carried out on this compound in filtered activated sludge solution. During the test period (approximately 6 months) no degradation products were observed.



(A)

Prior to undertaking biodegradation studies on a purified commercial stilbene-s-triazine FWA, Blankophor REU-P, a method was sought to determine its concentration in aqueous solution. Using ultraviolet absorbance measurements the concentration of the Blankophor could be determined in distilled water. However, this method was unsuitable when the Blankophor was dissolved in filtered activated sludge solution.
Attempts to extract the Blankophor from filtered activated sludge solution using solvent extraction and ion-exchange resins were unsuccessful.
Using computer modelling (SIMCA), combined with ultraviolet absorbance measurements, it was possible to determine the concentration of the Blankophor in filtered activated sludge solution. Biodegradation studies were undertaken with the Blankophor using this method to monitor the Blankophor concentration. During the test period (six days) the Blankophor concentration fell from 10ppm to approximately 5.5ppm. It was possible to deduce that the Blankophor was not being adsorbed onto the solid material of the activated sludge and that it had been metabolised and/or isomerised.
Two ion-pairing agents, tetraphenyl arsonium chloride and poly[oxyethylene(dimethylimino)-ethylene(diethylimino)-ethylene] were examined to try to separate the Blankophor and the metabolites and/or isomers from the filtered activated sludge solution but no successful separation was achieved. Thin layer chromatography was examined briefly but again with no success.
Photolysis experiments were undertaken with the Blankophor in distilled water. The concentration of the Blankophor fell from 10ppm to approximately 7.5ppm and its ultraviolet spectrum changed shape. This, coupled with the fact that the control spectra remained unaltered, indicated that the Blankophor had undergone photolytic conversion to metabolites and/or isomers.
Although it is clear that FWA's are fairly stable compounds which are slow to bio- and photo-degrade and that their toxicity to man, both directly and by bio-accumulationis is minimal little is still known about the toxicity of possible degradation products.

TABLE OF CONTENTS

INTRODUCTION

INTRODUCTION

Professional and public concern has grown during the past years over the chemical purity of water supplies. On the one hand there was a greater diversity of chemicals reaching rivers and streams because of the increasing production and usage of chemicals [this, however, has decreased because of the stringent requirements of the Sixth Amendment Directive of the Council of the European Communities 1981[1]], and on the other the water authorities have been forced to draw more and more on non ideal river sources to satisfy new and increased demands for water[2, 3, 4]. Professional concern has grown because of the availability of new analytical methods for the determination of trace levels of contaminants and because of a deeper knowledge not only of these compounds on health but also of their potential to bring about changes in human genetic processes[5, 6].

Since 1975 the Thames Water Authority has been assessing and controlling persistent low level contamination passed into rivers from industrial sources,[7, 8] and attention is now turning to discharges from domestic and allied premises,[9] such as pharmaceuticals, detergents, cosmetics, toiletries and disinfectants.

Both household detergents and photographic papers contain fluorescent whitening agents (FWA's). FWA's are substances which strongly absorb radiant energy in the near, ultraviolet region of the spectrum and re-emit it almost quantitatively in the form of longer wavelength visible blue light. In other words they function as light transformers. When they are applied to a material of a yellowish white colour, this blue light provides a brilliant whiter appearance by additive mixture.

On textiles a clean, unique white can thus be produced without using a chemical bleach (which might damage the fibre) and the overall cost of

the " Whitening " procedure greatly reduced. This explains why FWA's gained acceptance so soon after their discovery[10, 11]. They were quickly adopted by the textile, papermaking, plastics, synthetic fibres, and soap and detergents industries.

Since FWA's are used in surfactants they are certain to be present in domestic waste waters, and the first question therefore is how to determine this pollution load in a public sewage works. It is not easy to make a quantitative determination for various reasons:

(a) whiteners are used commercially in low concentrations (approx. 0.5%), and those of the stilbene type are liable to undergo trans-cis changes on exposure to light[12, 13]. Most methods of analyses involve fluorescence measurements and work carried out on behalf of The Thames Water Authority[14] indicates that much of the fluorescence is lost in the trans to cis change.

(b) several whiteners of similar or different types may be present at the same time[15].

(c) other naturally occurring fluorescent substances may also be present[16, 17].

Methods of analysis used in ecological tests are varied and a discussion of the advantages and disadvantages of some of the more common methods used can be found in: Fluorescent Whitening Agents, Environmental Quality and safety, Supplement Vol. IV[15].

Field trials carried out in the United States at a sewage treatment plant dealing with domestic and industrial effluents showed a reduction of more than 50% in whitener content at the primary purification stage[18]. At a sewage treatment plant dealing mainly with domestic waste water using a two-stage trickling filter system there was a reduction of

some 95% from the inlet to the outfall. At another two-stage purification plant with an activated sludge tank, dealing with a town effluent, a reduction of 83%[19] was found and 100% reduction is obtained by advanced treatment[19].

Tests carried out on activated sludge[20, 21] indicated that adsorption onto the activated sludge is the first stage of elimination of FWA and this has been confirmed by field trials at public sewage works[19, 22].

Numerous water sources have been examined for their FWA content. In the United States analyses were made of samples from rivers heavily polluted with industrial effluent, smaller rivers in non-industrial areas, and large and small lakes[19]. The quantities found were mostly <0.05 in 10^9. In another study, using improved methods of analysis, the average content in 58 samples was <1 in 10^9.

Many samples of drinking water have been analysed in Europe, the United States and Canada. In spite of a detection limit of 0.25 parts per thousand million no traces of FWA's has been found[17, 19].

During the months of June to August and September to December 1972 and again from January to April 1973, the river and drinking water at a number of points in seven European countries was examined for fluorescent content[23, 24, 25]. From these extensive studies of heavily contaminated rivers in Europe it was found that virtually no FWA's , or their known photo-conversion products, (excluding the cis isomers) were detected even by extremely sensitive methods of analysis. These results confirmed the earlier findings of Jensen and Petterson[22].

As previously noted most of the analysis involves fluorescence measurements and fluorescence can be lost by trans to cis isomerism. Hence, it was not surprising that no FWA's were found.

If traces of FWA's are present in river water they may be taken up by

fish directly from the water and indirectly through the food chain.

Feron and Hitz[26] carried out experiments to measure the uptake of a ^{14}C-labelled FWA by Golden Orfe from water and through a model food chain. Their results showed that there was no accumulation of the FWA by the direct route in the edible part of the fish, and that there was only slight accumulation (0.1%) in the non-edible parts. There was no metabolism of the FWA. Bioaccumulation through the food chain was found to be very slight. (<0.1%) The findings were confirmed by Hamburger et al[27] who carried out similar tests also on Golden Orfe.

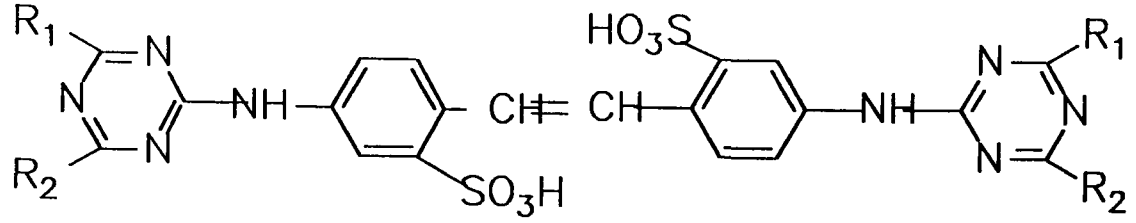
As a result of the increasingly critical attitude adopted by the authorities and the public towards the use of chemicals of all descriptions, more and more emphasis has been placed on the need to weigh benefit against hazard. In the case of products like FWA's, which are used to satisfy aesthetic criteria, particularly exacting demands are made with regard to safety, and neither the authorities nor the consumers are prepared to tolerate hazards attendant on their use.

The majority of FWA's are based on six major structural types. The stilbene type structures (I) are the most important in domestic usage[28] and only these structures will be considered in this investigation.

FWA's have been considered safe for the uses and exposure conditions for which they are intended. (see appendix 1. Toxicological details of FWA's)

As has been already mentioned a great deal of information is available concerning FWA's but little is known, however, about the biodegradability of the parent compounds or their primary degradation products, and there is concern that the many amino groups in the molecule could lead to metabolites exhibiting oncological effects, which might possibly be transferred to recycled waters.

The aims of this investigation are to study, using a wide range of physicochemical methods, the fate of stilbene-s-triazine FWA's in aquatic systems.



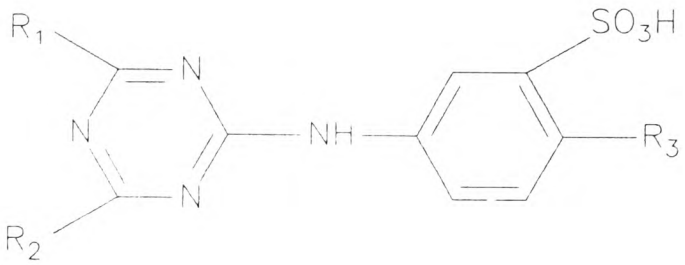
(1) STILBENE-S-TRIAZINE FWA

The groups R_1 and R_2 can be simple or complex amino groups.

Previous work on the fate of compounds of the type (I) has indicated that fluorescence is lost to a large extent due to a change from the trans to the cis isomer, and ^{14}C studies have suggested that the compound remains in the soluble phase[29].

Commercially available FWA's are not distinct compounds but a complex mixture, and the synthesis of discrete compounds of type (I) on a laboratory scale would be difficult. However, the most important question to be answered initially is whether removal of the sulphonate groups can occur readily under conventional sewage treatment processes, as the presence of sulphonate groups tends to detoxify many compounds[30].

Compounds of the type (II) are possible initial degradation products if degradation starts at the double bond, which is the most likely mechanism, and is supported by evidence from ^{14}C studies carried out for the Thames Water Authority.



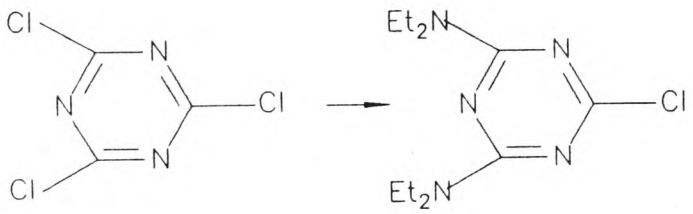
(II) MODEL COMPOUND USED IN BIODEGRADATION STUDIES

The initial part of this investigation was to synthesise a compound of the type (II) (where R_3 = $-CO_2H$ and R_1 = R_2 = NEt_2) to use as a model compound in biodegradation tests, under aerobic conditions, to determine whether desulphonation will occur, (in order to aid this investigation a model compound without the sulphonate group was synthesised) and to determine the type of metabolite which might be found.

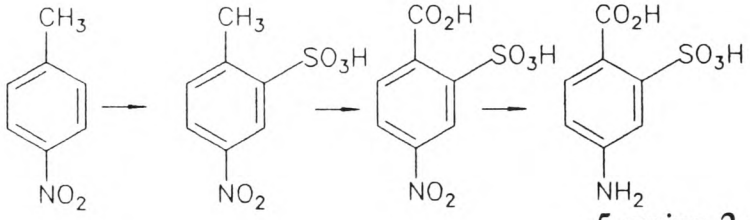
SYNTHESIS OF MODEL COMPOUND (II)

Synthesis of Model Compound (II)

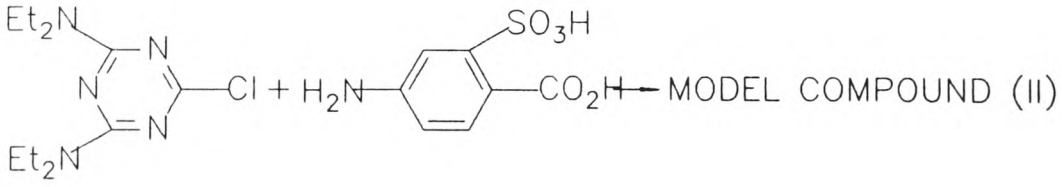
Synthesis of model compound (II) was envisaged as proceeding by the following route:



2,4-bis(diethylamino)-6-chloro-s-triazine



5-amino-2-carboxy-benzene sulphonic acid



Synthesis involved the difficult step of the reduction of the $-NO_2$ to the $-NH_2$ group, and then the coupling of the two separate molecules to obtain the model compound.

Reduction of the nitro group was attempted by the following methods:

1. Reduction using sodium sulphide and ammonium chloride: [31]

   Concentrated ammonium solution was added to an aqueous solution of the nitro compound and allowed to cool to 70^0C. Sodium sulphide was added in small (0.2g) aliquots and the mixture heated to 85^0 for thirty minutes under reflux conditions with constant stirring. The solution was filtered, evaporated to dryness, dissolved in boiling water , and then acidified with glacial acetic acid. Charcoal was added and the mixture boiled for one hour, filtered and evaporated to dryness. ^{13}C and ^1H N.M.R. spectra were obtained. The spectra indicated that a small quantity of product (<1%) had been formed but it was found to be impossible to purify, due to the small amounts present.

2. Reduction using stannous chloride and hydrochloric acid. [32]

   Nitro compound was added to a 25% solution of stannous chloride in concentrated hydrochloric acid sp. gr. 1.15, and the mixture heated under reflux conditions, with vigorous stirring, for two hours. The solution was basified with sodium hydroxide solution (2M) and the pH then adjusted to 10 using hydrochloric acid solution (2M) when a white organic precipitate appeared. The yellowish solution was filtered and evaporated to dryness to yield a yellowish solid. I.R. spectra indicated that the white material was inorganic and the yellow organic. ^{13}C and ^1H N.M.R. spectra showed that some product was present (approximately 5%) but that it was heavily contaminated.

Clean up techniques using solvent extraction with various solvents (ethanol, chloroform, methyl isobutyl ketone) were tried but to no avail.

3. Reduction by catalytic hydrogenation under pressure using Raney nickel.[33]

   Raney nickel (1g) was synthesised [34] and added to a 25% solution of the nitro compound in ethanol. The mixture was placed in a catalytic hydrogenator at 12 atmospheres, at $50^{0}C$, for 5.5 hours. The solution was filtered and the filtrate evaporated to dryness. The N.M.R. and I.R. spectra showed no relationship to those of the starting material or that expected for the product. After recrystalisation from boiling distilled water the spectra of the dried product indicated that the nitro compound had been partially reduced (approximately 5%). Further hydrogenation produced the desired product in reasonable quantities (approximately 80%).

Coupling the two separate compounds was attempted by the following methods:

1. Coupling using ethanol/water as a solvent. The two compounds were dissolved in an ethanol/water (50:50) solution and refluxed for 23 hours. The solution was filtered, evaporated to dryness, dissolved in distilled water, acidified with excess concentrated hydrochloric acid solution sp. gr. 1.15, and left to cool when a precipitate appeared. The mixture was filtered to yield a solid (a) and the filtrate evaporated to dryness to yield a solid (b). I.R. spectra of solids (a) and (b) indicated that no coupling had occurred.
2. Coupling using dimethyl sulphoxide as a solvent. The two compounds were dissolved in dimethyl sulphoxide (10g/l) and refluxed, on a boiling water bath, for 23 hours. After filtration and several clean

up stages (recrystallisation from water then ethanol)I. R. and N. M. R. spectra indicated that coupling had occurred but the yield was extremely low. (approximately 5%)

3. Coupling using dimethyl formamide as a solvent. The two compounds were dissolved in dimethyl formamide (10g/l) and heated under reflux conditions for ten hours. The solution was evaporated to yield an oily residue which, upon washing with hot water and recrystallisation from ethanol, gave the desired product in a reasonable yield. (40%)

The above investigations resulted in the following synthetic procedure being adopted.

(1). Preparation of 2,4-bis(diethylamino)-6-chloro-s-triazine [35].

As with the previous synthetic work, the following preparation was carried out in the fume cupboard due to the hazardous nature of the chemicals used. A drying tube containing anhydrous sodium carbonate was fitted to exclude moisture which is reactive with cyanuric chloride.

A solution of cyanuric chloride (73.6g, 0.4 moles), in tetrahydrofuran ($200cm^3$) was added to a stirred solution of diethylamine (204g), in tetrahydrofuran ($1000cm^3$), at 20^0C for one hour. The mixture was stirred for an additional eighty minutes. The tetrahydrofuran was then evaporated (rotary vacuum pump/60^0) to yield an oily residue. The oily residue was dissolved in trichloromethane and the trichloromethane extracted with water until the aqueous phase remained neutral. The organic layer was dried ($MgSO_4$), filtered, and evaporated to dryness (rotary vacuum pump/30^0). Crude 2,4-bis(diethylamino)-6-chloro-s-triazine was obtained. The crude product was purified by distillation under vacuum b.p. 138^0C/3mm, yield 53.3g

A sample of the purified product was sent to the Thames Water Authority

for a mass spectrum and for mutagenicity testing.

The following spectra were obtained:

^{1}H N. M. R.

^{13}C N. M. R. fully decoupled

I. R. spectra for the above compound and all following compounds are detailed in Appendix 3.

^{1}H and ^{13}C N. M. R. spectra for the above compound and all following compounds are detailed in Appendix 4.

Mass spectra for the above compound and all following compounds are detailed in Appendix 5.

Mutagenicity testing results for the above compound and all following compounds are detailed in Appendix 2.

(2). Preparation of 5-amino-2-carboxy-benzenesulphonic acid[36].

Stage 1.

4-Nitrotoluene (100g) was added very slowly (over a period of one hour) to oleum (200g) in a beaker (500cm^3) (FACE MASK AND GLOVES/FUME CUPBOARD) and warmed to 50^{0}C. The mixture was tested every ten minutes by adding a drop to water (1cm^3) until no precipitate appeared. Upon cooling, the mixture was poured slowly (over a period of thirty minutes) into ice (2000g) and calcium carbonate added until the mixture was neutral. The precipitated calcium sulphate was filtered off and potassium carbonate added until precipitation of calcium ceased. The solution was filtered and the filtrate evaporated (rotary evaporator/60^o) until crystallisation started. The solution was cooled, filtered and the precipitate dried to constant weight. Yield 92. 3g

^{13}C and ^{1}H N. M. R. spectra were obtained for both starting material and product.

Stage 2.

Product from stage 1. (2-sulphonic acid-4-nitro toluene 90g), together with potassium hydroxide (32.4g), was dissolved in water ($3000cm^3$), in a beaker ($5000cm^3$), and heated on a boiling water bath. When the solution had attained the temperature of the bath solid potassium permanganate (198g) was added and the solution left to heat until the colour of the permanganate had entirely disappeared. The amber coloured liquid was filtered and the dark brown residue thoroughly rinsed with hot water. ($500cm^3$) The filtrate and washings were then neutralised with hydrochloric acid solution (2M) and evaporated until the neutral potassium salt just began to precipitate out when a small aliquot of the solution was cooled. The remainder of the solution was allowed to cool, and concentrated hydrochloric acid (sp. gr. 1.15) added in excess, when the acid potassium salt of 4-nitro-2-sulphobenzoic acid separated out in fine white crystals.

Yield 60g

^{13}C and ^{1}H N.M.R. spectra of the product were obtained.

Stage 3.

Product from stage 2 (15g) was dissolved in ethanol and Raney nickel ($5cm^3$) added. The solution was placed in a catalytic hydrogenator, at twenty atmospheres, 50^0C, for fifty hours. The green coloured solution was filtered, to remove the Raney nickel, and the filtrate evaporated to dryness to yield a dark green solid. The solid was dissolved in hot water ($100cm^3$) and excess hydrochloric acid added. (pH meter) The solution was allowed to cool when the acid precipitated out. The solution was filtered and the solid dried to constant weight.

Yield 12.9g m.p. charred at 265^0C

A sample of product was sent to Thames Water Authority for mutagenicity

testing and for a mass spectrum, which indicated that the anhydride had been formed. The following spectra were also obtained:

^{13}C and ^{1}H N.M.R. spectra

I.R. spectrum

(3) Coupling 2,4-bis(diethylamino)-6-chloro-s-triazine with 5-amino-2-carboxy-benzenesulphonic acid.

5-Amino-2-carboxy-benzenesulphonic acid (3g) was added to a quick-fit conical flask followed by dimethyl formamide (DMF) ($250cm^3$). The solution was stirred (magnetic stirrer) and heated ($100-120^0C$) when the solid dissolved. (approximately 5min.) 2,4-bis-(Diethylamino)-6-chloro-s-triazine (2g) was added and the solution heated (under reflux conditions) with stirring for six hours. The solution was evaporated (rotary evaporator/60^O) to yield an oily residue. Water was added until a permanent precipitate appeared. The solution was allowed to settle, filtered and the solid washed thoroughly with acetone to remove all traces of the oil.

m.p. charred at 285^0C, yield 1.5g

N.M.R. and I.R. spectra were obtained and a sample of the product was sent to Thames Water Authority for a mass spectrum and for mutagenicity testing. The mass spectrum indicated that the anhydride had been formed.

(4) Preparation of 2,4-bis-(diethylamino)-6-(4-amino-carboxylic acid)-s-triazine.

2,4-bis(Diethylamino)-6-chloro-s-triazine (3g) was dissolved in DMF ($150cm^3$) in a Quick-fit conical flask and p-amino benzoic acid (3g) added. A condenser was fitted and the solution heated with stirring (hot plate/magnetic stirrer) for seventeen hours at 100^0C. The solution was evaporated (rotary evaporator/40^0) to yield an oily residue. Distilled

water was added until a permanent precipitate appeared. The precipitate was allowed to settle, filtered, and the solid washed thoroughly with acetone to remove all traces of the oil.

N.M.R. and I.R. spectra were obtained and a sample of the product sent to Thames Water Authority for mass spectrum, and for mutagenicity testing.

m.p. 202^{0}C, yield 2.1g.

BIODEGRADATION STUDIES WITH THE MODEL COMPOUND (II) IN ACTIVATED SLUDGE SOLUTION.

Biodegradation Experiments Using Activated Sludge:

Experiment 1.

Activated sludge ($200cm^3$) (supplied by The Welsh Water Authority Laboratory at Bridgend) was added to each of two measuring cylinders ($2000cm^3$) and distilled water added up to the $1000cm^3$ mark. The solutions were aerated at such a rate that any solid material remained in suspension. $3cm^3$ of each of the following nutrient solutions were added to each cylinder.

A:.... Potassium dihydrogen phosphate (8.50g).
Dibasic sodium phosphate dihydrate (33.40g).
Ammonium chloride (5.00g).
Dissolve in distilled water ($1000cm^3$).

B:.... Magnesium sulphate heptahydrate (22.5g).
Dissolve in distilled water ($1000cm^3$).

C:.... Calcium chloride dihydrate (36.40g).
Dissolve in distilled water ($1000cm^3$).

D:.... Ferric chloride hexahydrate (00.25g).
Dissolve in distilled water ($1000cm^3$).

The solutions were continuously aerated and allowed to stabilise (7 days) when aliquots ($25cm^3$) were taken from each cylinder (time T = 0). The aliquots were filtered, passed through a millipore filter (2 μm) and HPLC chromatograms obtained. (HPLC conditions are detailed in Appendix 6) The test compound (Model Compound II) (10mg) was added to the test cylinder and samples were taken periodically from both cylinders (see Figs 2.1, 2.2, 2.3) for HPLC analysis.

Nutrient solutions ($3cm^3$) were added every Monday, Wednesday and Friday. (the levels in the cylinders were maintained at $1000cm^3$ by addition of distilled water as required)

Before the addition of the test compound the chromatograms of both solutions were very similar (Fig 2.1 a,b). On addition of ten milligrams of the test compound a large peak appears with a retention time of approximately 2.3 mins. (Fig 2.1 c). This peak represents the test compound and can be seen more clearly (Fig 2.1 d) by subtracting the control chromatogram.

During the following four weeks the test compound peak gradually lessened in size (Fig. 2.2) indicating the removal of test compound from solution, either by adsorption onto the solid material, by metabolism , by removal in the sampling aliquots, or by a combination of all three. (may not have been using a large enough quantity of test compound but increasing this may prove to be lethal to the micro-organisms in the activated sludge)

The chromatograms show an indication of a second peak forming, prior to the test peak, but it is difficult to attribute this to a degradation product and may simply be the error due to subtracting the control chromatogram. It was decided to add more test compound to the test cylinder and to continue sampling as before.

Test compound was added at the following times:

T = 34 days..... 1mg

T = 40 days..... 2mg

T = 42 days..... 2mg

T = 79 days..... 5mg

The chromatograms (Fig. 2.3) still showed a steady decrease in the size of the test peak but did not show any further development of a second peak. It was thus decided to terminate this experiment and to start afresh examining both the aqueous and solid phases.

Fig. 2.1 HPLC Chromatograms

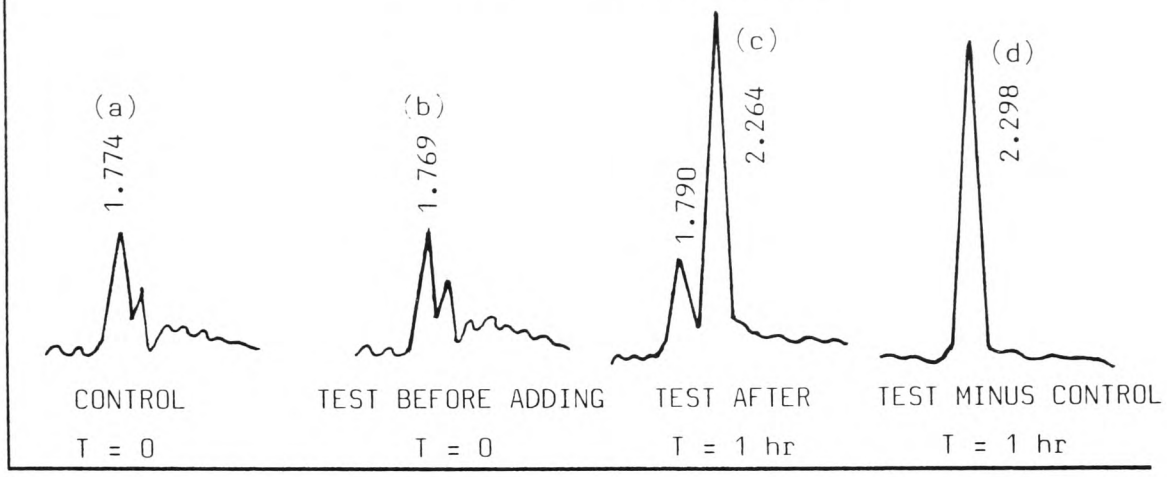


Fig 2.2 HPLC Chromatograms Test Minus Control

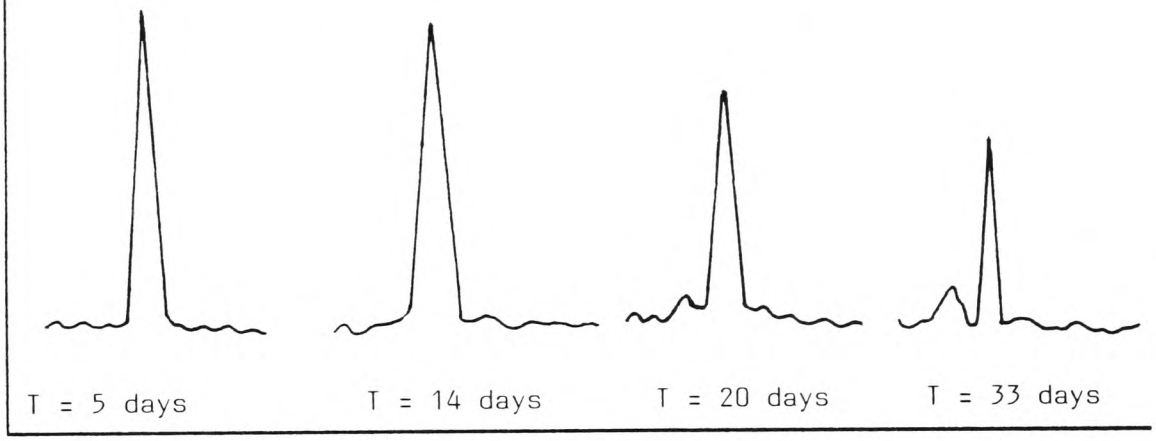
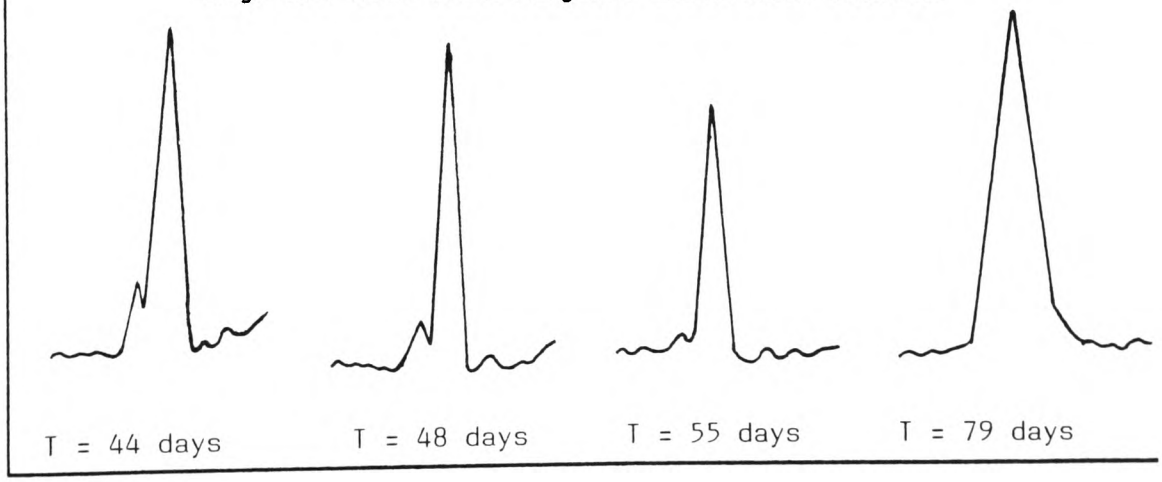


Fig. 2.3 HPLC Chromatograms Test Minus Control



Experiment 2:

Activated sludge ($200cm^3$) was added to each of two measuring cylinders ($2000cm^3$) and distilled water added up to the $1000cm^3$ mark. The solutions were aerated as before to ensure that any particulate matter remained in suspension. It was decided to feed the solutions with fresh activated sludge ($25cm^3$) every two weeks to improve bacterial growth, and to add the test compound in small doses over a period of time, to prevent sudden addition of a lethal dose.

The solutions were allowed to stabilise for one week when aliquots ($25cm^3$) were taken from each cylinder (T = 0). The aliquots were filtered and the solid materials (1) retained for further solvent extraction. The filtrates were passed through a millipore filter (2 μm) and HPLC chromatograms obtained. The solid materials (2) were extracted with chloroform ($25cm^3$), filtered, and the solid materials (2) retained for further extraction. The filtrates were passed through a millipore filter (2 μm) and HPLC chromatograms obtained. The solid materials were extracted with sodium hydroxide (2M, $25cm^3$) and then filtered. The filtrates were passed through a millipore filter (2 μm) and HPLC chromatograms obtained.

The test compound was added periodically (starting at T = 7 days) and aliquots taken (to be treated as above) as shown in table 2.1.

The chromatograms of the aqueous phase (Fig. 2.4a) show a steady decrease in the size of the test compound peak and an increase in its retention time and a development of a small secondary peak, at approximately 1.75 min, just prior to the test peak. Examination of the control chromatograms (Fig. 2.4b) show this secondary peak developing and increasing at approximately the same rate, indicating that it is not due to a degradation product of the test compound. Following the initial

Fig. 2.4 a HPLC CHROMATOGRAMS, TEST CYLINDER, AQUEOUS PHASE

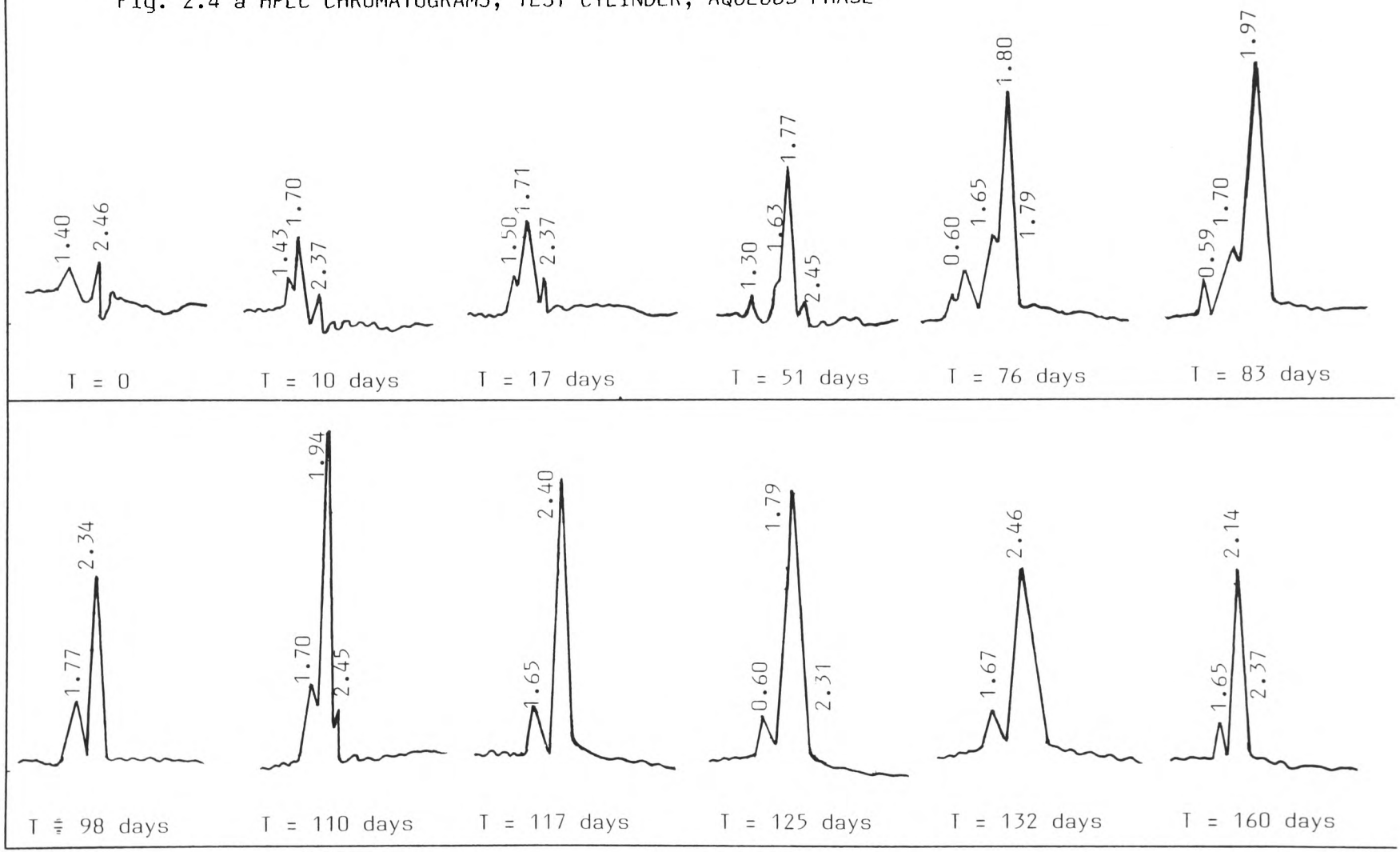
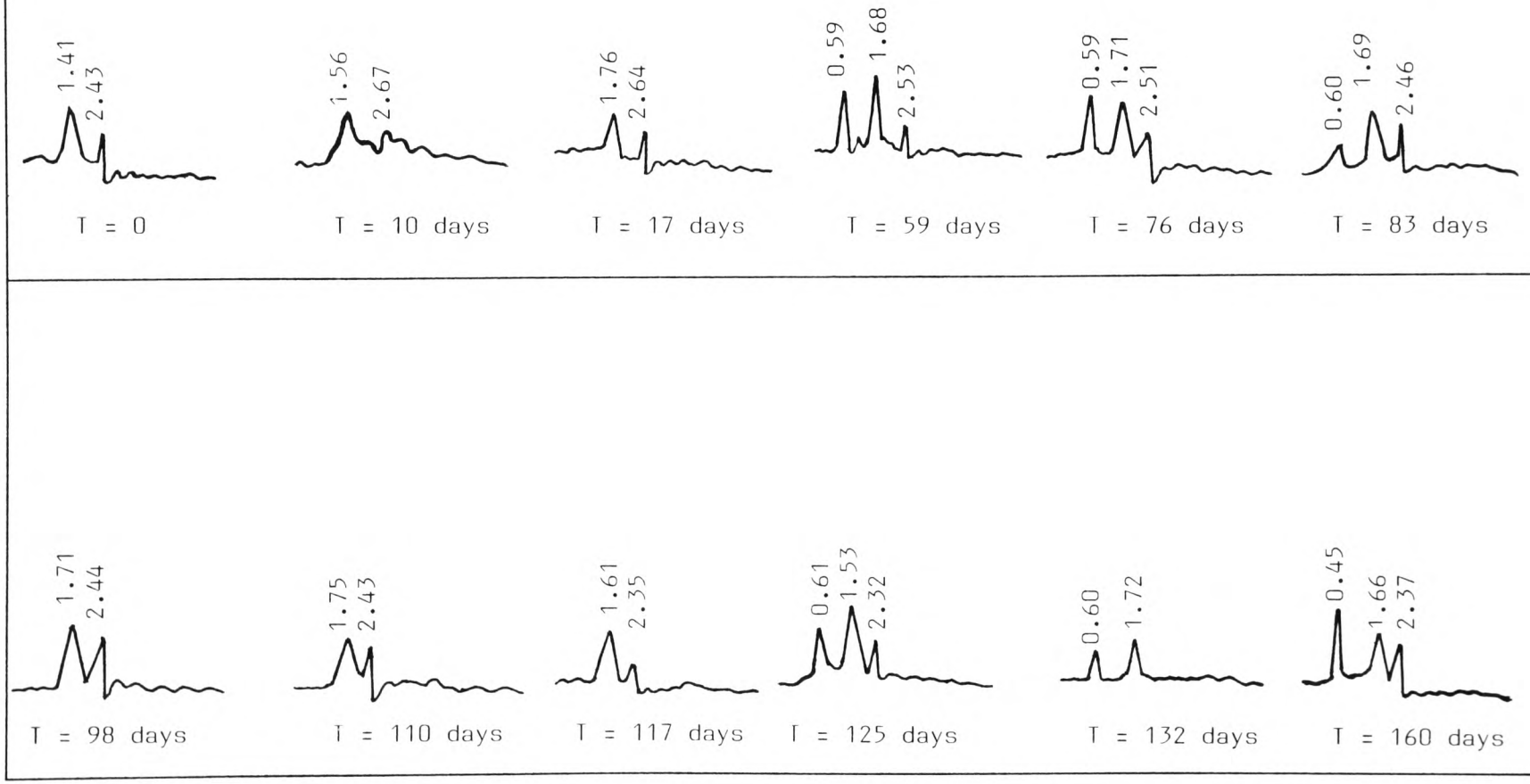


Fig. 2.4 b HPLC CHROMATOGRAMS, CONTROL CYLINDER, AQUEOUS PHASE



increase, after the addition of the last dose of test compound, the size of the test peak starts to increase.

TABLE 2.1

TIME SUBSTANCE ADDED	QUANTITY(mg)	TIME SAMPLES TAKEN
T = 7 days	0.75	T = 10 days
T = 13 days	1.45	T = 17 days
T = 24 days	1.45	T = 59 days
T = 59 days	1.50	T = 76 days
T = 77 days	1.25	T = 83 days
T = 83 days	1.50	T = 98 days
T = 98 days	3.50	T = 110 days
----------	----	T = 117 days
----------	----	T = 125 days

The chromatograms of the test chloroform phase (Fig 2.5 a) show only a chloroform peak between 4.5 and 5.0 min. There are only a few spurious peaks in several of the chromatograms but comparison with the control chromatograms (Fig 2.5 b) show no consistent peaks indicating the absence of any test compound or degradation products.

The chromatograms of the test sodium hydroxide phase (Fig 2.6 a) show peaks, of varying size, at approximately 1.5 and 2.5 min and in some of the later chromatograms a peak is seen to develop at approximately 3.0 min. The control chromatograms (Fig 2.6 b) also show the peaks at approximately 1.5 and to some extent the peak at 3.0 min. This may be an indication of some degradation having occurred but this is not definite. Apart from the slight possibility of a peak developing at approximately 3.0 min in the sodium hydroxide extracted phase there is no indication that any degradation has occurred.

Fig. 2.5 a HPLC CHROMATOGRAMS, TEST CYLINDER, CHLOROFORM PHASE

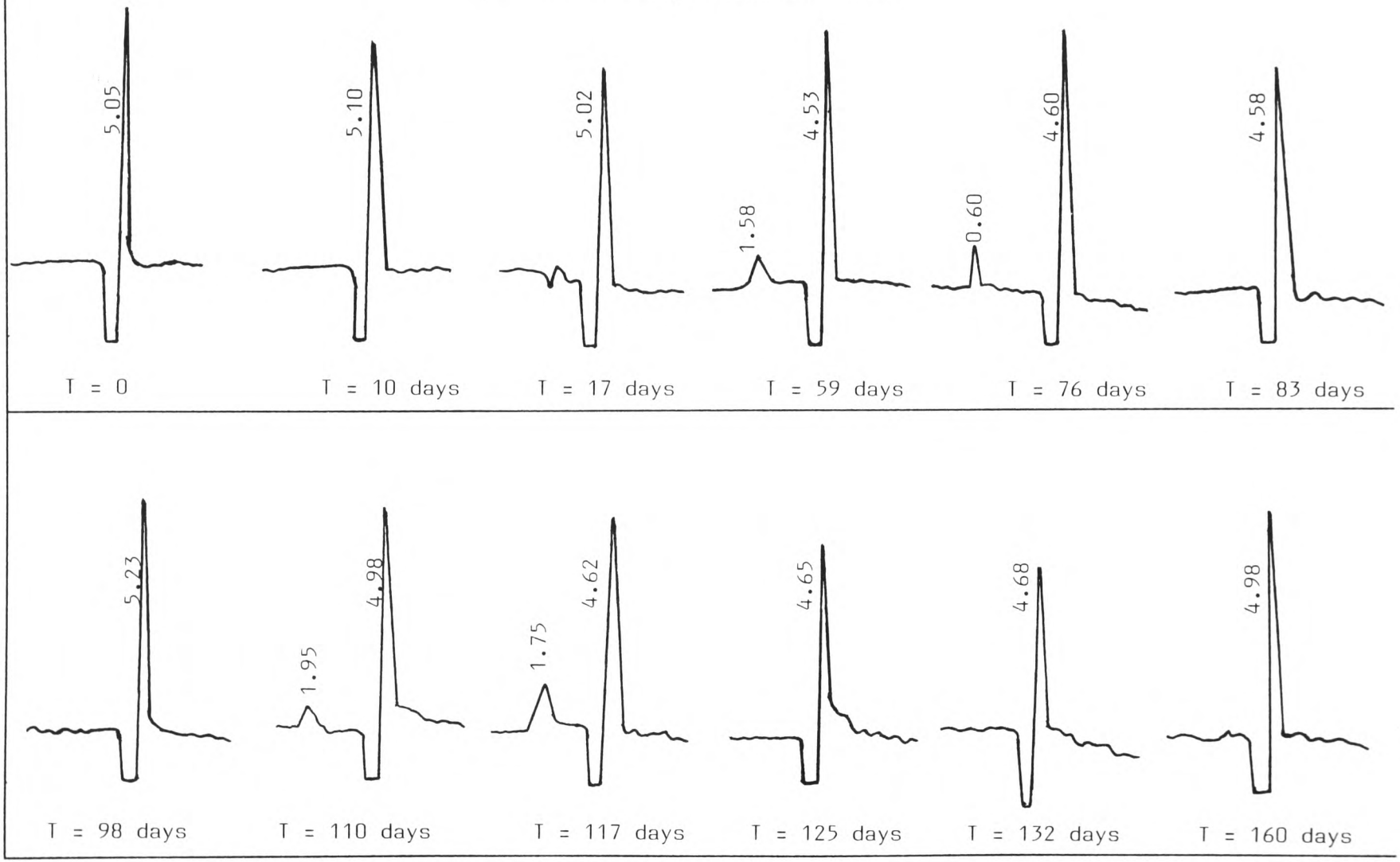


Fig. 2.5 b HPLC CHROMATOGRAMS, CONTROL CYLINDER, CHLOROFORM STAGE

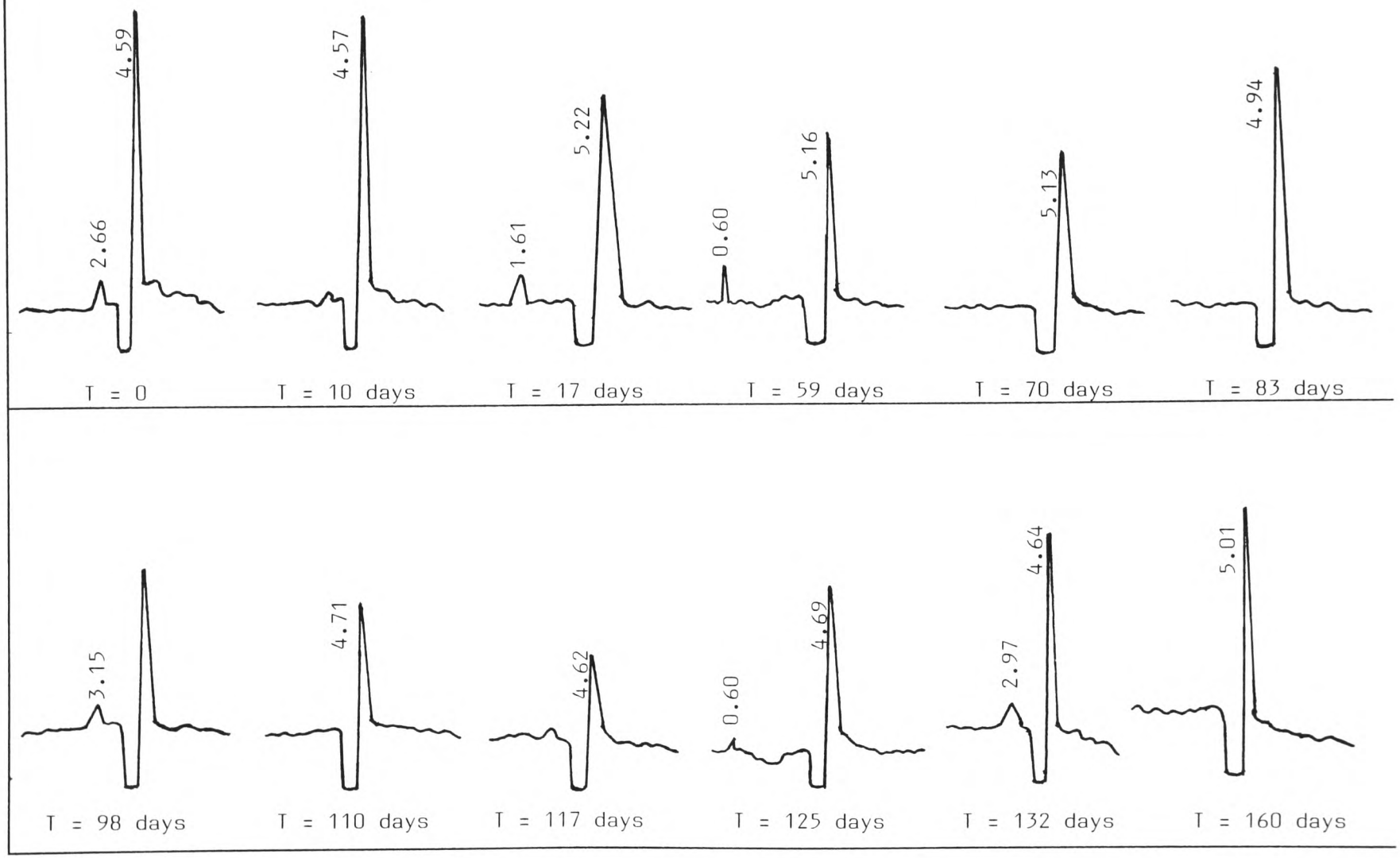


Fig 2.6 a HPLC CHROMATOGRAMS, TEST CYLINDER, SODIUM HYDROXIDE PHASE

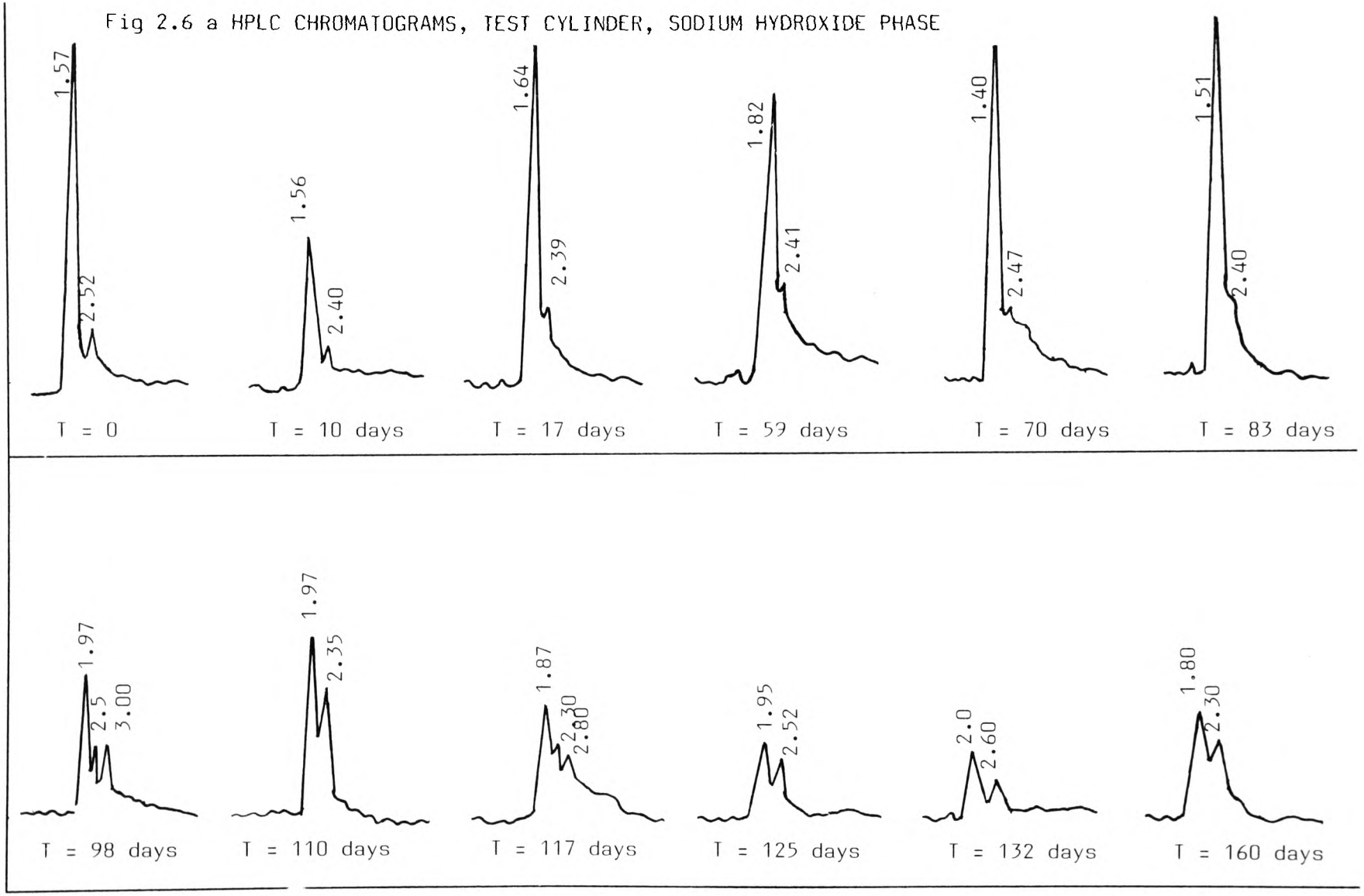
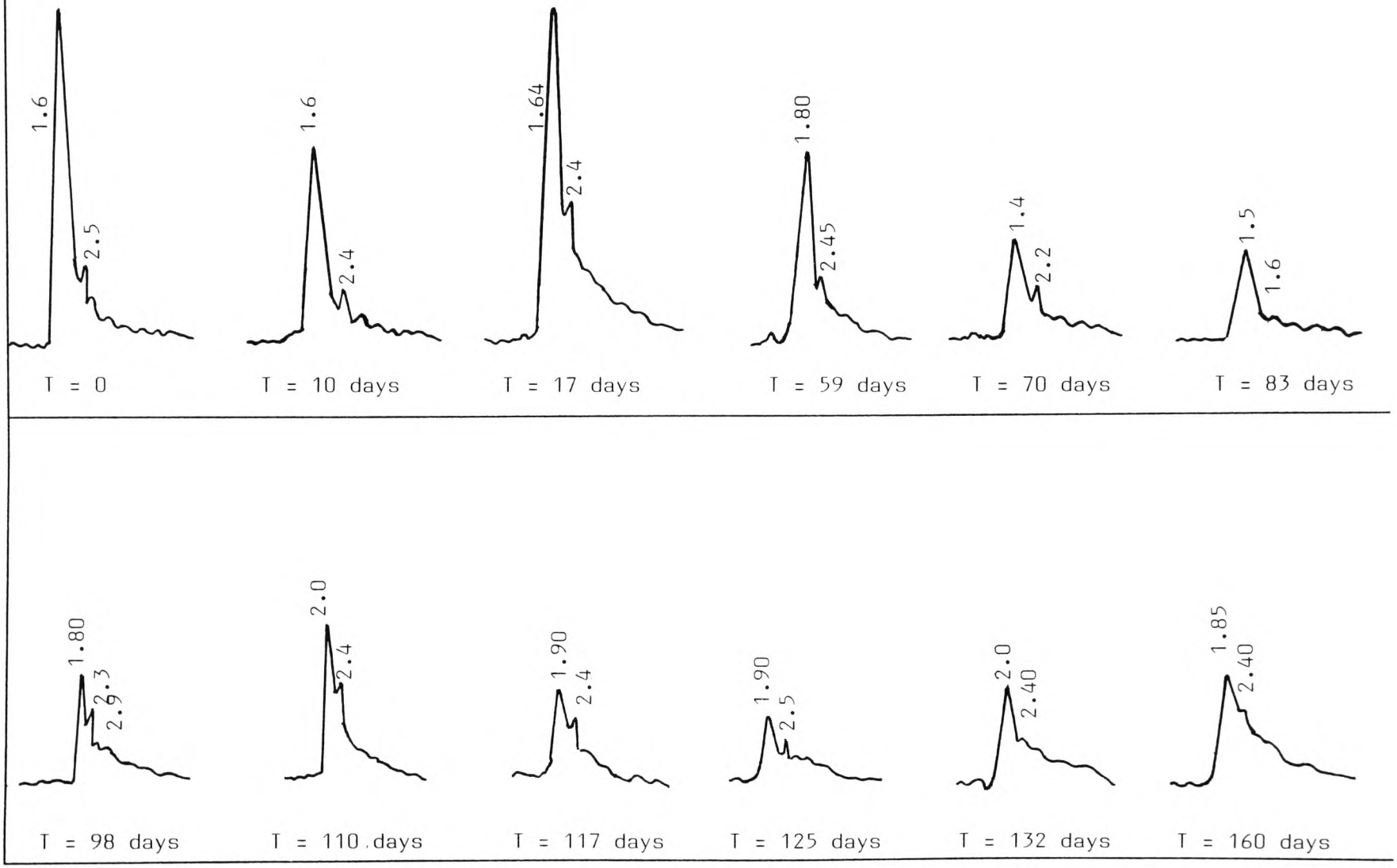


Fig. 2.6 b HPLC CHROMATOGRAMS, CONTROL CYLINDER, SODIUM HYDROXIDE PHASE



Extraction of the solid material with chloroform and sodium hydroxide solution did not yield any of the test compound indicating that no adsorption had occured. This, and the fact that there was no evidence of any degradation, suggests that the reduction in the size of the test peak may have been due to removal in the sample aliquots. However, if a mass balance is calculated only 1.5mg of Blankophor (out of a total of 11.4mg) should have been removed in the sample aliquots.

To see if the above results were reproducible another biodegradation experiment was undertaken.

Experiment 3.

The degradation of the test compound was carried out under the same conditions as the previous experiment and HPLC chromatograms were obtained (Figs 2.7, 2.8, 2.9). Table 2.2 shows when the test compound was added, how much was added, and when the samples were taken.

TABLE 2.2

TIME SUBSTANCE ADDED	QUANTITY/mg	TIME SAMPLES TAKEN
T = 7 days	0.75	T = 10 days
T = 14 days	1.50	T = 17 days
T = 24 days	1.50	T = 60 days
T = 60 days	1.50	T = 74 days
T = 76 days	1.30	T = 81 days
T = 83 days	1.45	T = 95 days
T = 98 days	4.00	T = 109 days
----------	----	T = 116 days
----------	----	T = 123 days
----------	----	T = 130 days
----------	----	T = 158 days

Fig 2.7 a HPLC CHROMATOGRAMS, TEST CYLINDER, AQUEOUS PHASE

Fig. 2.7 b HPLC CHROMATOGRAMS, CONTROL CYLINDER, AQUEOUS PHASE

Fig. 2.8 a HPLC CHROMATOGRAMS, TEST CYLINDER, CHLOROFORM PHASE

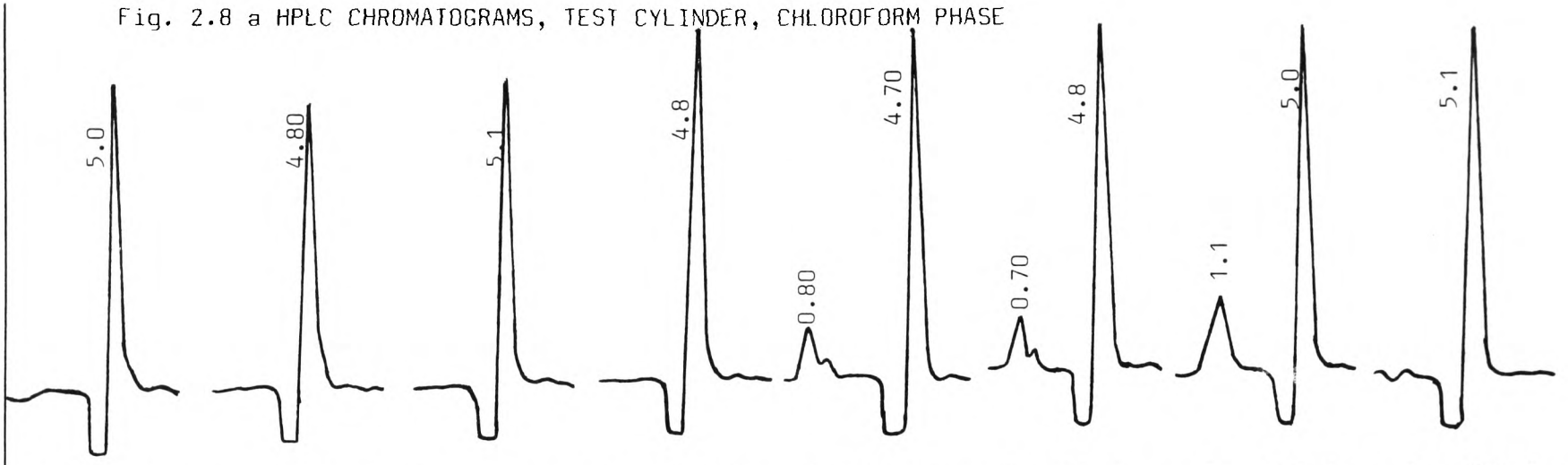


Fig. 2.8 b HPLC CHROMATOGRAMS, CONTROL CYLINDER, CHLOROFORM PHASE

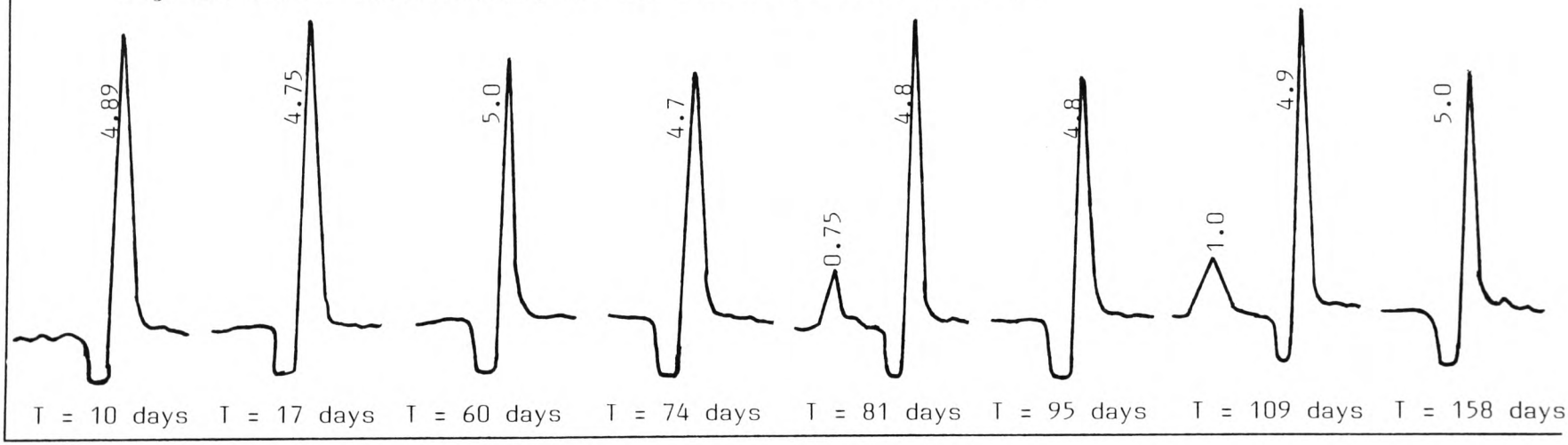


Fig. 2.9 a HPLC CHROMATOGRAMS, TEST CYLINDER, SODIUM HYDROXIDE PHASE

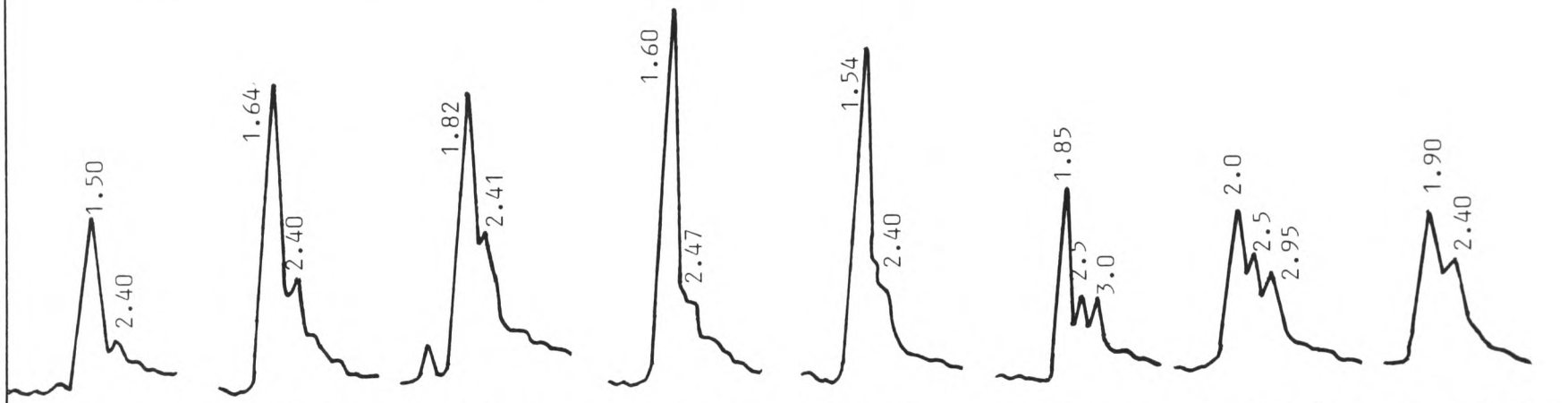
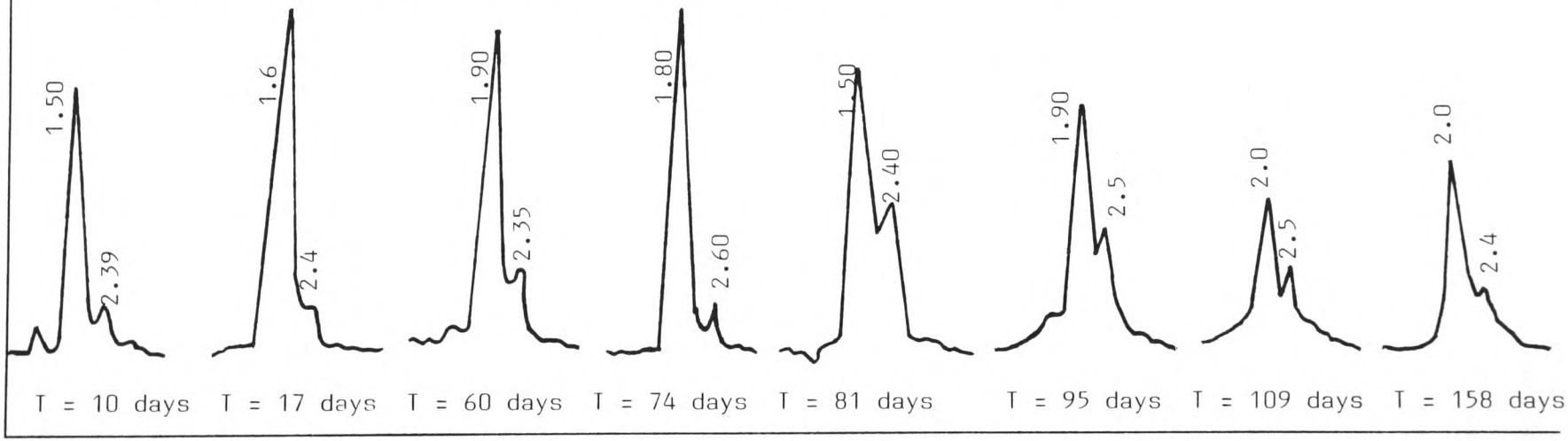


Fig. 2.9 b HPLC CHROMATOGRAMS,CONTROL CYLINDER, SODIUM HYDROXIDE PHASE



The Chromatograms obtained bear a marked similarity to those obtained in experiment 2, thus confirming its findings.

These experiments do not give any indication as to whether the sulphonic acid group can be readily removed.

To determine if the HPLC system was capable of separating the test compound from the compound without the sulphonic acid group HPLC chromatograms were obtained of the following solutions:

(a)... Pure test compound (5mg/1000cm^3) (Fig. 2. 10 a)

(b)... Pure compound without the sulphonic acid group (5mg/1000cm^3) (Fig 2. 10 b)

(c)... Mixture (50: 50) of (a) 5mg/1000cm^3 and (b) 5mg/1000cm^3 (Fig 2. 10 c).

Examination of the spectra show that the HPLC system and conditions used (see Appendix 6.) were not capable of separating the two compounds (variuos solvent mixtures and solvent gradients were tried but with no success). This was not surprising because of the similarity of the two compounds. Examination of their ultraviolet spectra (Fig 2. 11) (the HPLC detector is ultraviolet) also shows a marked similarity.

As the I. R. spectra (Appendix 3) of the two compounds are different, infra-red spectroscopy was considered as a possible means of identifying the two compounds in any future degradation experiments. However, due to the high I. R. absorption of water, it was not possible to obtain aqueous I. R. spectra even when using an FT-I. R. spectrophotometer.

Because of the inability of the HPLC system to separate the two compounds it was decided to abandon the PLC analysis and postpone any further degradation studies until an analytical method could be found to determine the two compounds.

At this time the Thames Water Authority were able to supply a purified

sample of a stilbene-s-triazine type FWA (BLANKOPHOR REU-P). It was decided that all further studies should be carried out using the latter pure commercial FWA. A method was thus sought for the determination of the Blankophor REU-P FWA.

Henceforth the purified stilbene-s-triazine Blankophor REU-P FWA will be referred to as Blankophor unless otherwise stated.

Fig. 2.10 HPLC Chromatograms

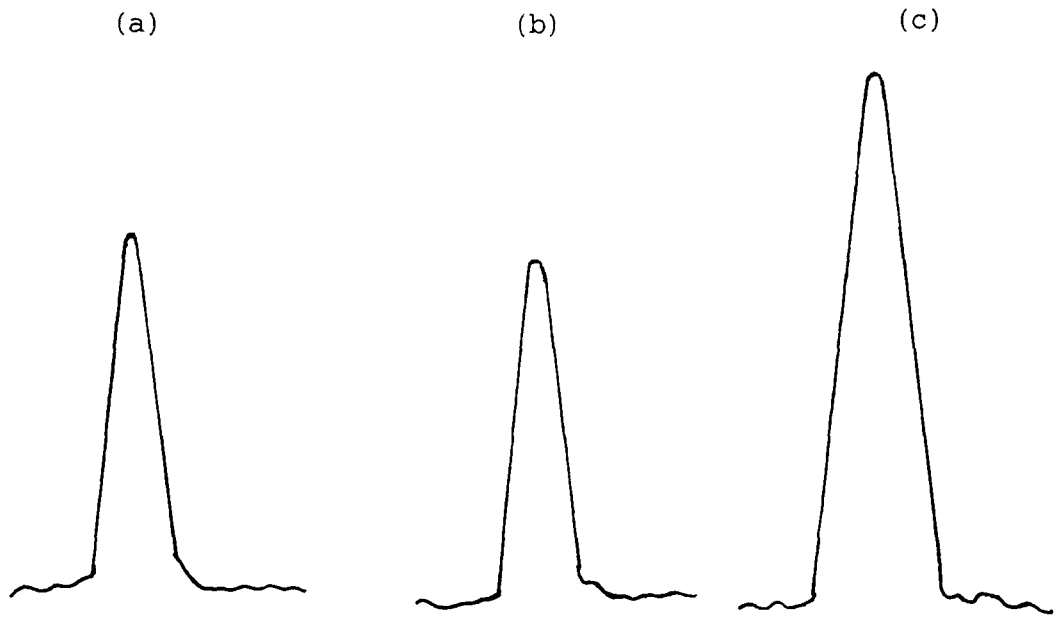
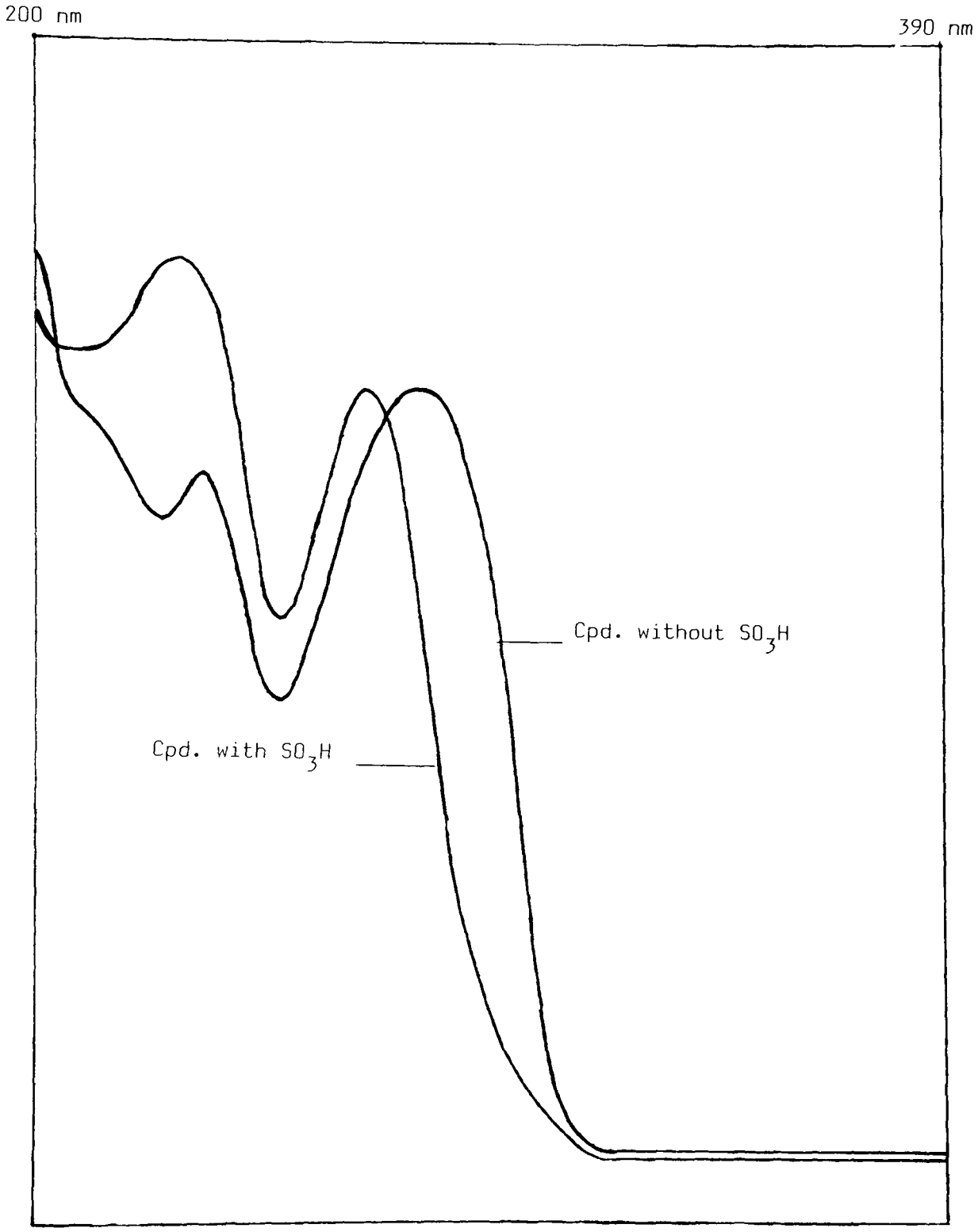


Fig. 2.11 Ultraviolet Spectra



DETERMINATION OF BLANKOPHOR REU-P PURIFIED COMMERCIAL FWA.

Determination of Blankophor REU-P Purified Commercial FWA (Blankophor) in Aqueous Solutions.

Stilbene FWA's absorb strongly in the ultra violet region of the electromagnetic spectrum and fluoresce strongly in the blue region of the spectrum. Because of these characteristics it was decided to investigate ultra violet and fluorescence spectroscopy as possible methods to determine Blankophor in aqueous solution.

The fluorescence of various standard (0-20ppm) distilled water solutions of Blankophor were measured (excitation wavelength 320nm) at various wavelengths (320-450nm). Unfortunately the readings bore no relationship to the concentrations of the Blankophor solutions and this technique was immediately discounted as an analytical method for Blankophor determination.

Standard solutions (0-20ppm) of Blankophor were made up in distilled water and absorbance readings measured at various wavelengths (Table 3.1). The spectrophotometer (Perkin Elmer λ3) was zeroed with distilled water in both the reference and sample cells. Graphs of Blankophor concentration versus absorbance readings were constructed for each wavelength (Figs. 3.1 to 3.8).

The plots were linear with the absorbance readings showing a high degree of correlation with the concentrations of the Blankophor solutions. Two further sets of Blankophor solutions were made up in distilled water and absorbance readings taken as before (Tables 3.2 and 3.3). These readings were very similar to those in Table 3.1 indicating that this was a good method for determining Blankophor concentration in aqueous solutions.

TABLE 3.1 Ultraviolet Absorbance Measurements.

Blankophor conc. ppm	Wavelength/nm 210	220	230	240	270	280	320	330
0.0	0.005	0.073	0.005	0.010	0.010	0.007	0.010	0.000
2.0	0.101	0.120	0.107	0.080	0.045	0.058	0.064	0.075
4.0	0.223	0.217	0.217	0.161	0.065	0.079	0.127	0.160
6.0	0.338	0.337	0.332	0.238	0.130	0.143	0.183	0.225
10.0	0.560	0.558	0.549	0.399	0.209	0.224	0.309	0.381
12.0	0.680	0.673	0.659	0.481	0.254	0.267	0.369	0.460
14.0	0.793	0.778	0.765	0.561	0.286	0.304	0.433	0.540
16.0	0.912	0.891	0.872	0.643	0.329	0.349	0.496	0.620
20.0	1.142	1.092	1.073	0.810	0.363	0.393	0.635	0.805

TABLE 3.2 Ultraviolet Absorbance Measurements.

Blankophor conc. ppm	Wavelength/nm 210	220	230	240	270	280	320	330
0.0	0.010	0.075	0.010	0.007	0.010	0.007	0.009	0.005
2.0	0.105	0.120	0.110	0.081	0.040	0.057	0.065	0.070
4.0	0.220	0.217	0.219	0.160	0.065	0.080	0.127	0.160
6.0	0.340	0.340	0.330	0.240	0.130	0.140	0.185	0.230
10.0	0.560	0.560	0.550	0.390	0.210	0.225	0.310	0.381
12.0	0.680	0.675	0.660	0.480	0.255	0.270	0.370	0.460
14.0	0.795	0.780	0.765	0.561	0.280	0.305	0.435	0.540
16.0	0.920	0.890	0.870	0.645	0.330	0.351	0.495	0.620
20.0	1.145	1.101	1.075	0.810	0.360	0.395	0.635	0.810

TABLE 3.3 Ultraviolet Absorbance Measurements.

Blankophor con. ppm	WAVELENGTH/nm 210	220	230	240	270	280	320	330
0.0	0.000	0.070	0.007	0.008	0.010	0.008	0.008	0.000
2.0	0.100	0.120	0.110	0.079	0.042	0.060	0.064	0.080
4.0	0.220	0.220	0.208	0.160	0.060	0.080	0.130	0.160
6.0	0.340	0.340	0.330	0.240	0.135	0.140	0.190	0.235
10.0	0.560	0.555	0.550	0.385	0.214	0.230	0.315	0.385
12.0	0.685	0.680	0.659	0.485	0.260	0.275	0.375	0.460
14.0	0.800	0.780	0.765	0.560	0.280	0.300	0.440	0.540
16.0	0.925	0.891	0.870	0.650	0.335	0.350	0.500	0.625
20.0	1.150	1.100	1.070	0.815	0.368	0.399	0.640	0.810

U.V. ABSORBANCE READINGS VERSUS BLANKPHOR CONCENTRATION (DISTILLED WATER SOLUTION).

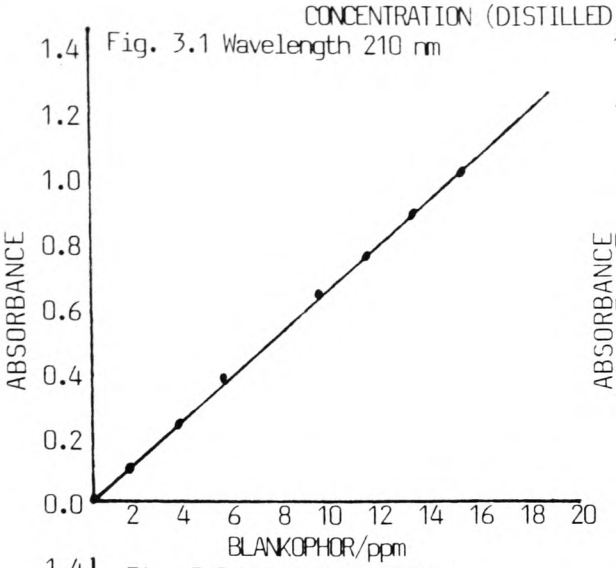


Fig. 3.1 Wavelength 210 nm

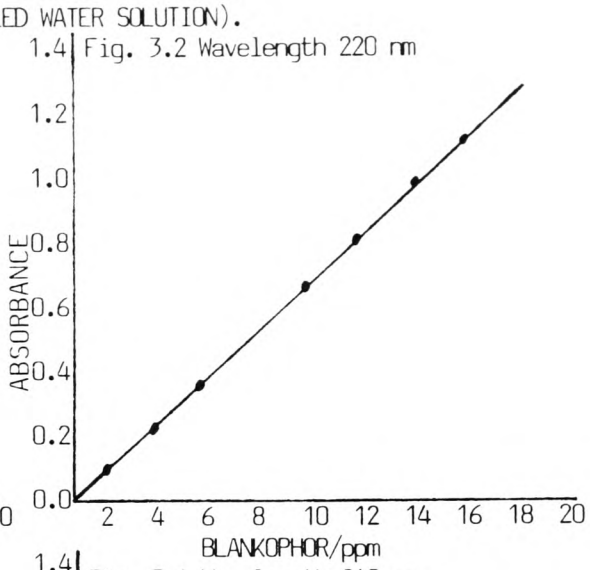


Fig. 3.2 Wavelength 220 nm

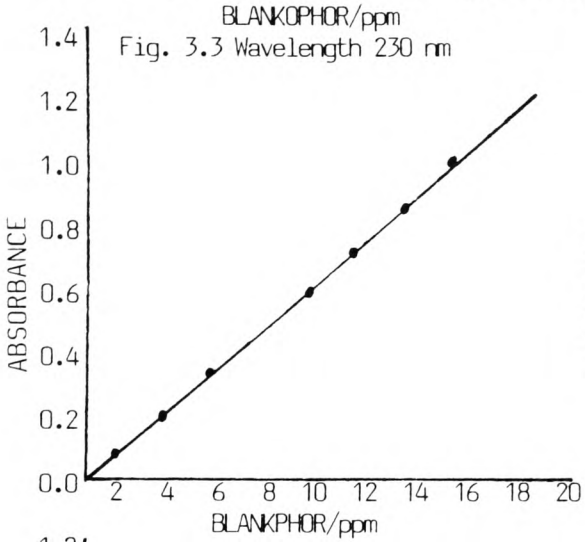


Fig. 3.3 Wavelength 230 nm

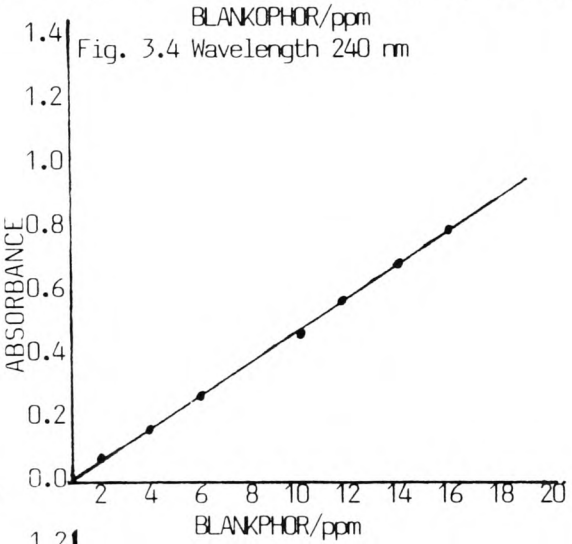


Fig. 3.4 Wavelength 240 nm

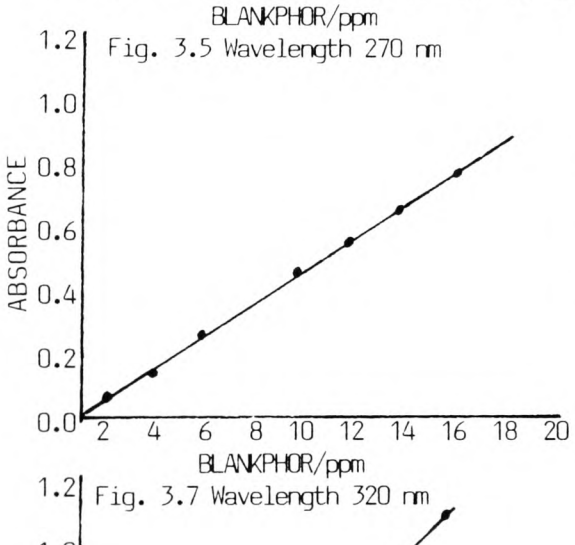


Fig. 3.5 Wavelength 270 nm

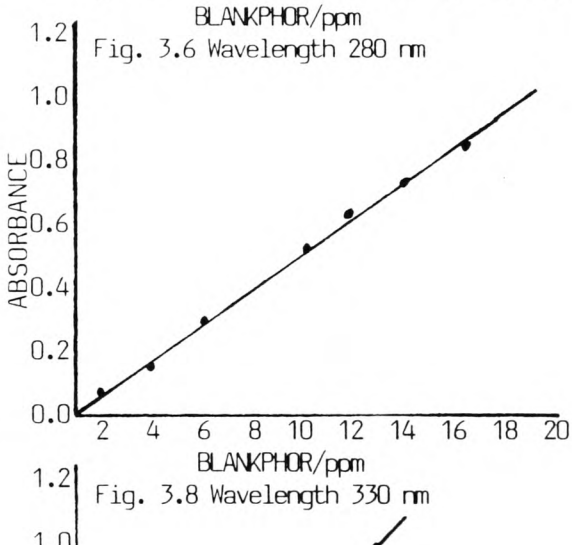


Fig. 3.6 Wavelength 280 nm

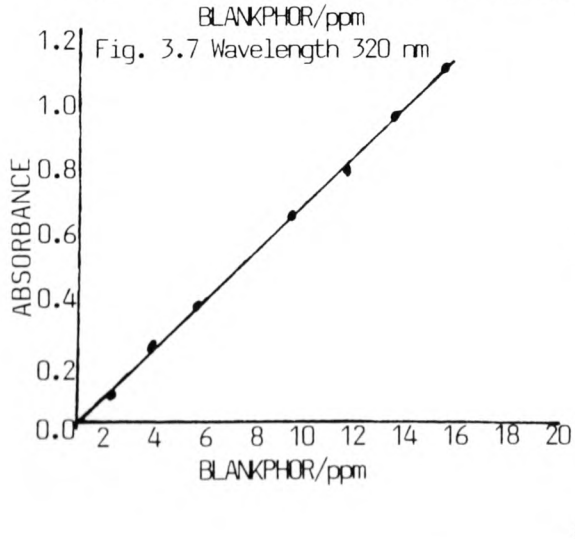


Fig. 3.7 Wavelength 320 nm

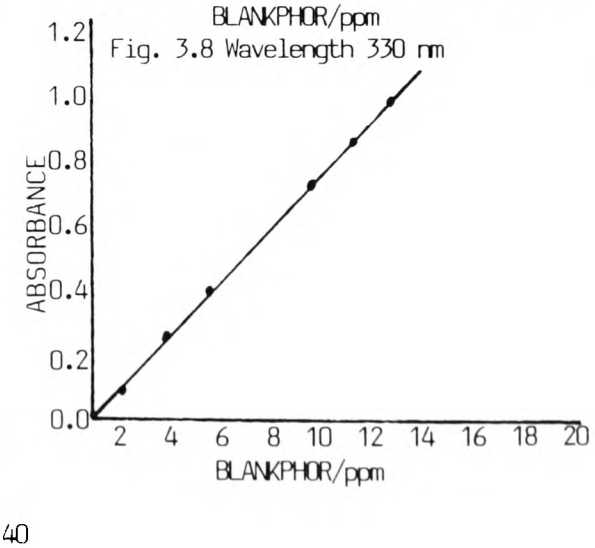


Fig. 3.8 Wavelength 330 nm

Before undertaking any biodegradation experiments with the Blankophor, using ultra violet absorbance measurements as a means of determination, it was decided to see if the matrix of filtered activated sludge solution interfered with the analysis.

Filtered activated sludge solution was prepared as follows: -

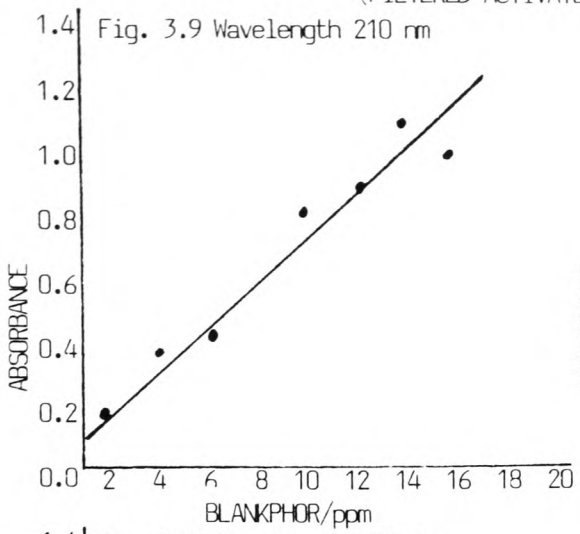
Unfiltered activated sludge ($200cm^3$) was made up to $1600cm^3$ with distilled water, aerated (24hrs) and then filtered (Whatman N^O1 then Whatman N^O44 ashless).

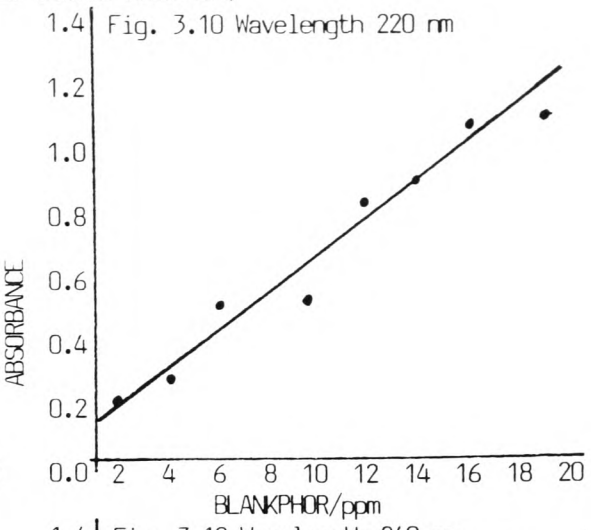
Standard solutions of Blankophor (0 to 20ppm) were made up in the filtered activated sludge solution and absorbance readings measured at various wavelengths (Table 3.4). The ultra violet spectrophotometer (Perkin Elmer λ3) was zeroed with filtered activated sludge solution in both reference and sample cells. Graphs of Blankophor concentration versus absorbance readings were constructed for each wavelength (Figs. 3.9 to 3.16).

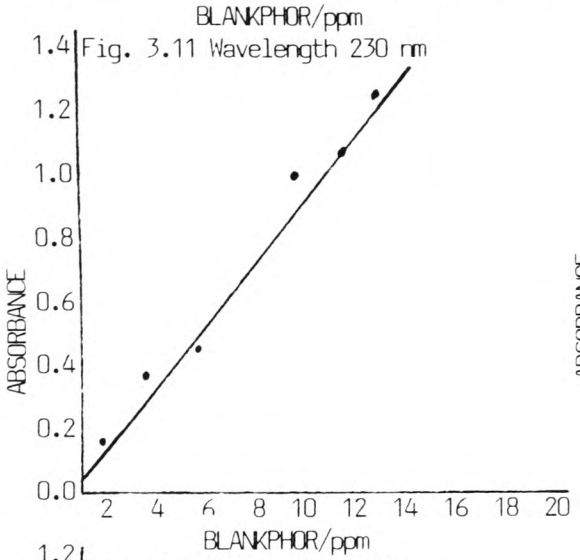
TABLE 3.4

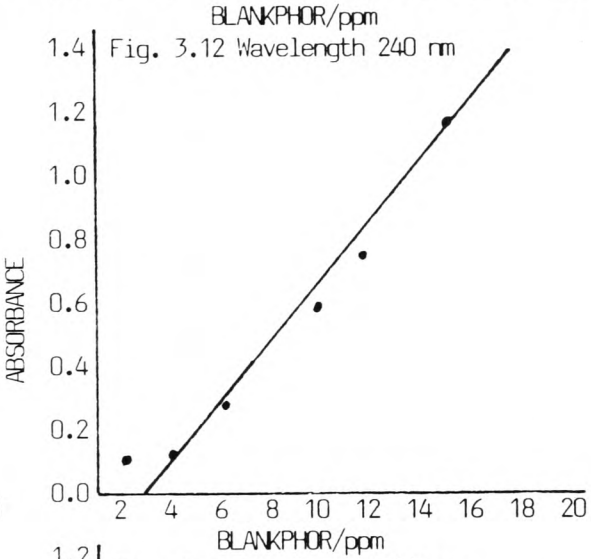
Blankophor conc. ppm	Wavelength/nm 210	220	230	240	270	280	320	330
2.0	0.200	0.200	0.100	0.060	0.070	0.060	0.080	0.090
4.0	0.340	0.240	0.120	0.070	0.110	0.140	0.090	0.120
6.0	0.380	0.480	0.140	0.110	0.120	0.120	0.090	0.070
10.0	0.670	0.500	0.340	0.220	0.280	0.320	0.160	0.190
12.0	0.710	0.720	0.360	0.290	0.270	0.270	0.170	0.210
14.0	0.916	0.740	0.580	0.480	0.340	0.320	0.280	0.340
16.0	0.820	0.880	0.620	0.500	0.380	0.340	0.300	0.380
20.0	1.100	0.900	0.780	0.690	0.340	0.360	0.440	0.560

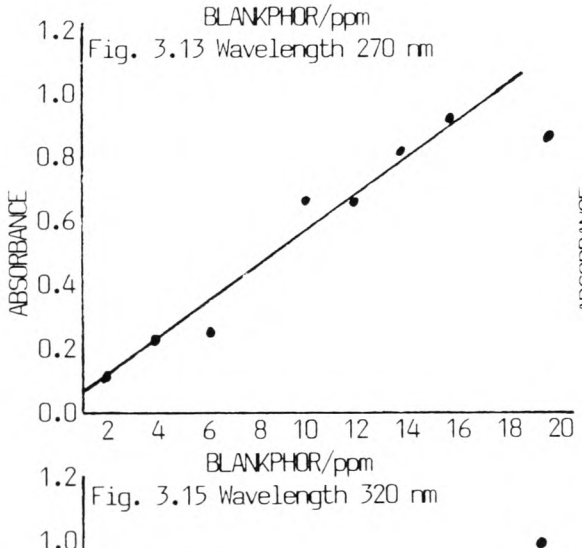
U.V. ABSORBANCE READINGS VERSUS BLANKPHOR CONCENTRATION
(FILTERED ACTIVATED SLUDGE SOLUTION)

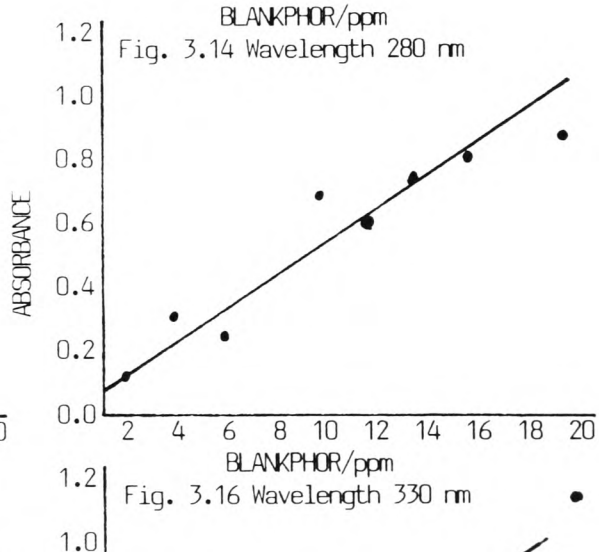


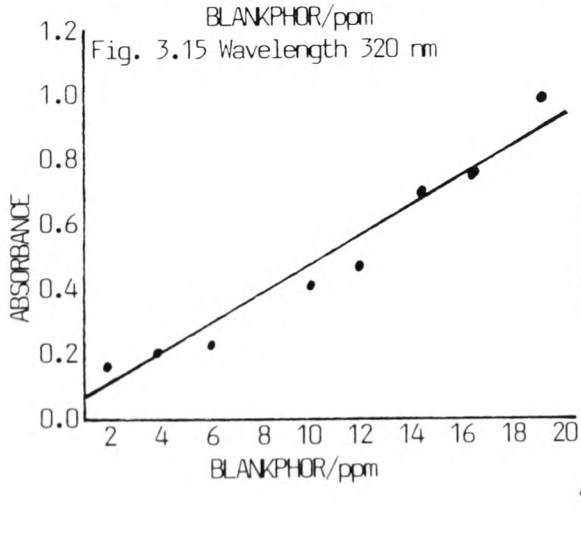


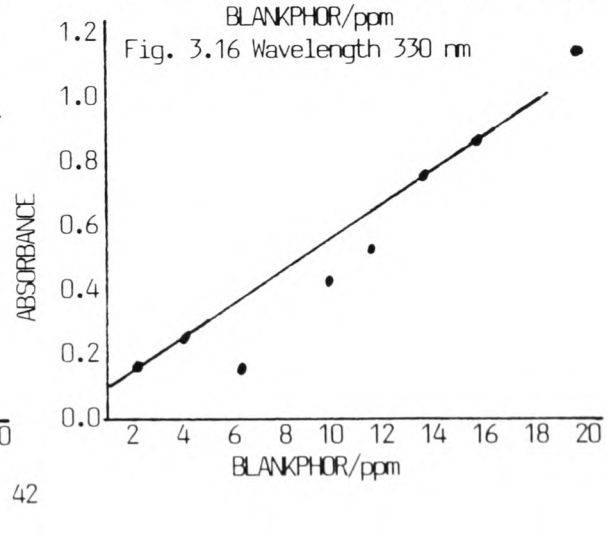












With the Blankophor in filtered activated sludge solution there was only a poor to fair correlation between Blankophor concentration and absorbance readings.

It was decided to repeat this experiment using the following solutions:

(a) Fresh filtered activated sludge solution prepared from the same batch of activated sludge.

(b) Filtered activated sludge solution prepared from a new batch of activated sludge.

(c) Fresh filtered activated sludge solution prepared from the same activated sludge as (b).

It can be seen from Tables 3.4 to 3.7 that the correlation between Blankophor concentration and absorbance readings varies greatly from one batch of activated sludge to another and indeed within the same batch.

The difference between batches was not unexpected as sewage treatment plants are not necessarily processing exactly the same materials at any given time. The difference within batches was more surprising. It may be that the composition of the sludge solution changed (by biodegradation/photodegradation) between experiments, or there may have been some desorption of compounds from the solid materials.

Several more batches of activated sludge were obtained and filtered activated sludge solution prepared. Standard solutions of Blankophor were made up in these filtered activated sludge solutions and ultra violet absorbance measurements taken as before.

Again there was no reproducibility of results within and between batches. It was decided to seek a clean-up procedure to separate the Blankophor from the complex filtered activated sludge solution prior to ultraviolet determination of concentration.

TABLE 3.5 Ultraviolet Absorbance Measurements.

Blankophor conc. ppm	Wavelength/nm 210	220	230	240	270	280	320	330
2.0	0.63	0.40	0.20	0.10	0.11	0.07	0.08	0.09
4.0	0.94	0.64	0.23	0.12	0.14	0.14	0.10	0.13
6.0	1.10	0.68	0.26	0.19	0.20	0.13	0.10	0.10
10.0	1.40	0.74	0.48	0.33	0.32	0.31	0.20	0.23
12.0	1.75	0.96	0.50	0.38	0.41	0.29	0.21	0.22
14.0	1.90	0.98	0.69	0.60	0.53	0.34	0.29	0.43
16.0	1.90	1.20	0.74	0.70	0.69	0.34	0.35	0.41
20.0	2.40	1.36	0.89	0.84	0.88	0.38	0.48	0.62

TABLE 3.6 Ultraviolet Absorbance Measurements.

Blankophor conc. ppm	Wavelength/nm 210	220	230	240	270	280	320	330
2.0	3.10	3.20	2.30	0.68	0.23	0.22	0.14	0.13
4.0	3.00	2.80	1.40	0.44	0.19	0.18	0.11	0.10
6.0	3.00	2.80	1.44	0.48	0.24	0.23	0.14	0.14
10.0	2.90	2.40	1.24	0.48	0.30	0.29	0.18	0.17
12.0	2.80	2.20	1.18	0.50	0.34	0.33	0.20	0.20
14.0	2.50	1.96	1.11	0.51	0.37	0.36	0.22	0.21
16.0	2.20	1.71	1.04	0.51	0.40	0.39	0.24	0.24
20.0	1.60	1.23	0.87	0.53	0.34	0.34	0.33	0.37

TABLE 3.7 Ultraviolet Absorbance Measurements.

Blankophor conc. ppm	Wavelength/nm 210	220	230	240	270	280	320	330
2.0	3.00	2.10	0.87	0.56	0.14	0.14	0.10	0.10
4.0	2.90	2.40	1.11	0.84	0.15	0.10	0.11	0.14
6.0	2.80	2.50	1.44	0.84	0.11	0.11	0.08	0.16
10.0	3.00	2.50	1.69	0.93	0.21	0.20	0.14	0.14
12.0	2.50	2.90	2.30	0.90	0.26	0.25	0.16	0.18
14.0	2.90	3.00	2.30	1.20	0.34	0.32	0.21	0.21
16.0	2.00	3.00	2.70	1.40	0.38	0.37	0.23	0.22
20.0	1.50	3.10	2.90	1.50	0.36	0.34	0.38	0.42

EXTRACTION OF BLANKOPHOR FROM FILTERED ACTIVATED SLUDGE SOLUTION.

Extraction of Blankophor REU-P from Filtered Activated Sludge solution.

1. Solvent Extraction.

Dichloromethane, methyl iso-butyl ketone (MIBK), ethyl acetate and chloroform were examined to see if the Blankophor (or the interfering species) could be extracted from the filtered activated sludge solution In each case a standard solution of blankophor ($100cm^3$, 10ppm) in filtered activated sludge solution was continuously extracted with each solvent separately ($250cm^3$) under reflux conditions (24 hours). The layers were separated and the organic layer was dried (anhydrous magnesium sulphate) and filtered. The aqueous layer was evaporated to dryness to exclude all traces of solvent and the solid residue dissolved in distilled water ($100cm^3$).

Absorbance readings were measured at various wavelengths (210 to 330nm), for each solvent extraction, for the following solutions:

(a) Standard Blankophor solution (10ppm) in filtered activated sludge solution,

(b) Each solvent,

(c) Dried organic layer after continuous extraction,

(d) Reconstituted aqueous layer after continuous extraction.

In each case a distilled water blank was treated as per the standard Blankophor solution.

With all four solvents examined neither the Blankophor or any of the unknown interfering species were extracted from the filtered activated sludge solution.

2. Ion-Exchange Resin Extraction.

The following ion-exchange resins were examined to see if the Blankophor (or the interfering species) could be extracted from the filtered

activated sludge solution: -

(a) Sep-Pak cartridges: both silica and C18 types supplied by Waters Associates.

(b) Extrelut ART11737: prepacked resin columns for extraction of lipophilic compounds from bodily fluids, supplied by BDH Chemicals Ltd.

(c) Amberlyst 27 - A/27: a strongly basic macroreticular anion exchange resin, active group $N(CH_3)+$, used in the chloride form, supplied by BDH Chemicals Ltd.

Standard solutions of Blankophor (100ppm) in distilled water, and filtered activated sludge solution, were passed through each type of column to see if either the blankophor or the interfering species were retained on the columns.

Both types of Sep - Pak cartridges retained neither the Blankophor or the interfering species.

The Extrelut retained both the Blankophor and the interfering species only slightly. (approximately 5%)

The Blankophor and the interfering species were retained totally by the Amberlyst 27 resin columns.

Because of the strong retention of both the Blankophor and the interfering species by the Amberlyst resin it was decided to examine the possibility of separating the Blankophor from the interfering species by the following procedures: -

(a) washing the column with increasing quantities of distilled water prior to elution with 2M hydrochloric acid solution,

(b) loading the columns at various pH's,

(c) eluting the columns at various pH's.

In all cases the Amberlyst 27 columns were prepared as follows: -

The resin was allowed to swell in a beaker of distilled water for twenty four hours. The resin was washed with sodium chloride solution (4 by 25 cm^3 2M) to prepare the chloride form. The resin was then washed with distilled water until the wash liquor was neutral (pH meter) and 10 cm^3 poured into a glass column (15cm^3 by 1cm^3 internal diameter) and back washed with distilled water to eliminate air bubbles and pack the columns evenly.

(a) Washing the column with increasing quantities of distilled water prior to elution with 2M hydrochloric acid solution.

Five columns were loaded with standard Blankophor solution (25cm^3, 10ppm) made up in filtered activated sludge water. Columns 1, 2, 3, 4 ,5, were washed with 25, 50, 75, 100, and 125cm^3 of distilled water respectively, eluted with hydrochloric acid solution (2M, 25cm^3) into volumetric flasks (25cm^3) and absorbance readings measured at various wavelengths. (Table 4. 1)

To check the reproducability of the results the above procedure was repeated. (Table 4. 2)

TABLE 4. 1 Ultraviolet Absorbance Measurements.

	Wavelength/nm									
solution	210	220	230	240	270	280	320	330	340	350
2MHCl	.001	.008	.014	.018	.018	.020	.021	.024	.025	.125
Column 1.	1.069	.917	.787	.671	.417	.436	.643	.682	.658	.582
Column 2.	1.042	.880	.747	.637	.388	.406	.622	.666	.649	.576
Column 3.	1.121	.921	.757	.634	.388	.405	.610	.646	.626	.555
Column 4.	1.134	.945	.786	.662	.409	.428	.630	.665	.641	.564
Column 5.	0.980	.858	.747	.641	.378	.394	.622	.668	.654	.582

TABLE 4.2 Ultraviolet Absorbance Measurements.

	Wavelength/nm									
Solution	210	220	230	240	270	280	320	330	340	350
2M HCl	0.000	.008	.010	.014	.020	.020	.018	.020	.085	.120
Column 1.	1.071	.920	.787	.674	.421	.437	.640	.684	.660	.584
Column 2.	1.047	.894	.767	.642	.398	.416	.624	.669	.652	.578
Column 3.	1.120	.925	.760	.637	.384	.415	.612	.652	.630	.564
Column 4.	1.125	.920	.786	.672	.414	.430	.632	.665	.651	.560
Column 5.	1.025	.875	.767	.652	.409	.410	.622	.668	.654	.582

Examination of Tables 4.1 and 4.2 show that washing the column with varying quantities of distilled water prior to elution with 2M hydrochloric acid solution does not aid separation of the Blankophor from the interfering species.

(b) Loading the columns at various pH's.

The pH of various standard Blankophor solutions ($25cm^3$, 10ppm) made up in filtered activated sludge solution, were adjusted (sodium chloride, 2M/hydrochloric acid solutions, 2M) to 2, 3, 6, 8, 10, 11, and 13. The solutions were passed through the columns and collected in volumetric flasks ($25cm^3$) for absorbance measurements. (Table 4.3) The columns were eluted with hydrochloric acid solution (2M, $25cm^3$) into volumetric flasks and absorbance readings measured at various wavelengths. (Table 4.3)

In each case a blank ($25cm^3$ of distilled water with pH adjusted as for the test solutions) was carried out.

Examination of Table 4.3 indicates that, at all the pH's examined, the Blankophor and the interfering species were both totally retained on the column.

TABLE 4.3 Ultraviolet Absorbance Measurements.

BP = blank passed through column;

TP = test passed through column;

BE = blank eluted;

TE = test eluted;

		Wavelength/nm								
pH	Solution	210	220	230	240	270	280	320	330	340
2	BP	.115	.100	.089	.074	.029	.030	.043	.064	.063
2	TP	.125	.097	.092	.081	.034	.033	.051	.061	.063
2	BE	.114	.104	.090	.070	.040	.040	.035	.054	.064
2	TE	1.550	1.14	1.00	.860	.590	.630	.630	.670	.640
3	BP	.040	.032	.010	.014	.008	.009	.004	.004	.004
3	TP	.050	.030	.009	.009	.004	.004	.002	.003	.002
3	BE	.100	.130	.000	.002	.001	.001	.120	.010	.010
3	TE	1.515	1.14	.893	.746	.502	.533	.551	.586	.559
6	BP	.000	.000	.001	.002	.007	.007	.004	.000	.000
6	TP	.001	.003	.003	.006	.009	.009	.005	.002	.000
6	BE	.120	.160	.090	.100	.110	.110	.210	.090	.010
6	TE	1.563	1.30	1.08	.907	.661	.706	.647	.657	.489
8	BP	.000	.000	.000	.001	.001	.001	.001	.001	.001
8	TP	.001	.001	.000	.003	.003	.003	.000	.001	.000
8	BE	.160	.023	.100	.070	.090	.100	.200	.010	.000
8	TE	1.700	1.41	1.05	.862	.616	.657	.615	.632	.584
10	BP	.048	.029	.009	.009	.004	.005	.001	.001	.000
10	TP	.050	.032	.010	.010	.002	.003	.002	.002	.003
10	BE	.000	.030	.001	.002	.001	.001	.190	.010	.010
10	TE	1.321	1.11	.962	.862	.574	.612	.600	.627	.587
11	BP	.061	.054	.020	.010	.005	.005	.003	.001	.001
11	TP	.087	.043	.018	.005	.003	.003	.002	.000	.001
11	BE	.400	.450	.430	.300	.300	.400	.400	.200	.200
11	TE	1.990	1.56	1.37	1.16	.850	.910	.810	.810	.720
13	BP	.061	.035	.042	.019	.021	.008	.009	.006	.002
13	TP	.071	.040	.054	.021	.018	.002	.004	.004	.003
13	BE	.300	.300	.210	.140	.100	.120	.240	.100	.120
13	TE	1.720	1.29	1.15	.970	.650	.690	.680	.710	.650

(c) Eluting the columns at various pH's.

The columns were loaded with standard solutions of Blankophor ($25cm^3$, 10ppm) made up in filtered activated sludge water. The columns were eluted with solutions whose pH's were adjusted to 2.0, 3.0, 6.0, 7.0, 8.0, 10.0, 11.0, and 13.0. (using sodium chloride 2M/hydrochloric acid solutions, 2M) Absorbance measurements were taken as in the previous experiment.

For the pH range 2.0 to 6.0 and 8.0 to 13.0 there was total elution of both the Blankophor and the interfering species. At pH 7.0 there was no elution of either species.

In the absence of a suitable " clean up " procedure a method was sought that would effectively mask the interfering species. A computer modelling programme (SIMCA) was examined to see if the concentration of the Blankophor could be determined in the filtered activated sludge solution.

SIMCA MODELLING.

Determination of Blankophor Concentration in Filtered Activated Sludge Solution using SIMCA Computer Modelling.

Introduction:

In traditional analytical techniques one relies on measuring one single variable, eg. the light absorbance at a single wavelength. To avoid errors due to interferences one therefore has to purify and standardise the samples first, in order to get sufficiently specific measurements. This is often time-consuming, and in some cases the required purification is too expensive or even physically impossible.

By multivariate data analysis one can use measured data more efficiently. Multivariate calibration can reduce the need for sample preparation in chemical analysis, because various systematic noise types can be eliminated mathematically. Reliable quantitative measurement therefore becomes possible even in "dirty" systems.

Multivariate analysis and computer modelling processes using these techniques involve complex mathematical and statistical principles. It is beyond the scope of this discussion to give a detailed mathematical and statistical explanation of multivariate analysis [there is a wide range of literature available on this topic [37, 38, 39]. It is intended only to show how SIMCA was used in the determination of Blankophor in filtered activated sludge solution.

Data Analysis:

Traditionally, analytical chemists have concerned themselves with the steps of making measurements and relating them to chemical information using very straight forward calibration methods. The microprocessor revolution has enabled chemists to acquire and store great quantities of data easily and cheaply. Instead of merely generating data, analytical chemists are becoming chemical problem solvers and are using

multivariate data analysis methods to uncover the meaning of the chemical information they produce.

Given an n-dimensional data set, that is , a set of samples with n measurements made on each sample, the aim is to learn something about a property or behaviour, answer a question, or test a hypothesis about the system. The type of property or question to be investigated influences the selection of data analysis techniques that are appropriate to use. Generally, data analysis problems are one of two types: involving either category data or continuous property data. It is the latter type of data analysis problem which is encountered in the determination of Blankophor by the measurement of ultraviolet absorbances at various wavelengths.

A continuous property is a dependent variable associated with a sample that has a continuous range of possible values. An example of a data analysis technique appropriate for this case would be multivariate linear regression where the continuous property (the dependent variable) is related to a set of measurements (the independent variables).

SIMCA uses a method of continuous property data analysis called Partial Least Squares (PLS) path modelling.

The notion of scaling is intuitive to anyone who has ever plotted some points on a piece of graph paper. Typically, the criteria for scaling are to fill the page and to show about equal magnitude of variation in the points on each axis so it "looks right." However in data analysis problems, a more rigorous approach to scaling is needed.

In the absence of any prior information the data should be scaled so as to put all the variables on an equal footing in terms of their variance. As an example of the problems encountered with unscaled data, consider a plot of bond length versus heat of fusion for a set of molecules. In

terms of absolute numerical magnitude, the variation in bond length is very small in comparison to the variation in the heat of fusion. Due to our arbitrary choice of units, the latter variance would obscure the information contained in the bond length variable, which could be significant even though small.

Auto scaling removes any inadvertent weighting that arises due to arbitrary units. Auto scaling to unit variance refers to mean-centering followed by dividing by the standard deviation on a variable by variable basis. The variance of an autoscaled feature is equal to 1.0.

SIMCA uses auto scaling to remove any inadvertent weighting that arises due to arbitrary units.

In general, a set of co-ordinate axes may be rotated through an angle θ to change the relative orientation of a set of points to the axes. As a preprocessing step, it is extremely useful to rotate all the axes involved in an n-dimensional data set so that the first new axis corresponds to the direction of greatest variance in the data, and each successive axis represents the maximum residual variance. Without changing the data structure one wants to find orthogonal (independent) axes to represent the directions of maximum variance.

The axes are rotated mathematically and eigenvalues (variances associated with each new axis) obtained and the eigenvectors associated with them determined. Eigenanalysis is useful in modelling the data, for example, each category may be modified by one or more of its eigenvectors and a classification allogorithm can be developed.

SIMCA uses such a procedure as a basis for modelling data.

SIMCA:

The name SIMCA may be thought of as an acronym for Soft Modelling of Class Analogy.

In the SIMCA method, a principal component model is fitted to each category and confidence envelopes (volumes or hypervolumes) are constructed around the model to contain the data points. The closed class envelope for each category is derived by first doing a principal component analysis separately on each class. The number of principal components used in the model may be pre-selected or determined by cross-validation. If one principal component were used, the data would be modeled by the class mean and a line (the first principal component). If two principal components were used, the class mean and a plane would form the model.

In the determination of Blankophor using SIMCA the number of principal components used is the model is determined by cross-validation.

Cross-validation is performed by an iterative procedure in which elements are first removed from the data matrix; usually 10% of the data is deleted in a prescribed manner. Beginning the iteration with the number of principal components equal to one, a principal component analysis is performed on the reduced data set. The deleted data values are predicted using these principal components.

Of course, using less than the truly significant number of principal components will mean that the prediction will be out by an amount E. These errors are calculated and stored as the procedure is repeated 100/%deleted times, changing the deletion pattern position so that each point is left out once and only once. A quantity called the PRESS (predictive residual error sum of squares) is calculated and stored for later use.

The number of principal components is increased by one and the entire procedure is repeated. As the number of principal components approaches the true number of significant components, the prediction should improve and thus the PRESS should decrease. However, as the truly significant number is passed, we begin to include noise within the model, which certainly has poor predictive ability. Thus the PRESS should start to increase again at this point. Hence we can make a good estimate of the true number of significant components.

Before considering any further biodegradation studies the following trial study was carried out to see if SIMCA could be used to determine Blankophor concentration in filtered activated sludge solution:

Activated sludge ($200cm^3$) was added to each of five measuring cylinders ($2000cm^3$) and distilled water added up to the $1000cm^3$ mark. The solutions were aerated continuously for 24 hours and activated sludge solution prepared from them by filtering the solutions, Whatman number 1, then Whatman number 44 ashless.

Four standard solutions of Blankophor (10, 8, 6, and 4ppm) were made up in the filtered activated sludge water prepared from cylinder number one, to be used as unknowns (P1, P2, P3, and P4 respectively) in the SIMCA modelling.

A set of standard Blankophor solutions (0, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5 and 20ppm) were made up in the filtered activated sludge water from each of the remaining four cylinders, to be used as knowns (A1 to A9, B1 to B9, C1 to C9, and D1 to D9 respectively) in the SIMCA modelling.

Ultra-violet absorbance readings were taken, for all the above solutions, at fourteen equally spaced wavelengths from 210 to 340nm, and the data (Table 5.1) modeled using SIMCA.

TABLE 5. 1

Ultraviolet Absorbance Readings of Blankophor Solutions at Various Wavelengths from 210 to 340nm.

Wavelength/nm (Absorbance readings in $A/10^{-3}$,														CONC. PPM	OBJECT NAME
0	3	6	6	7	8	8	8	8	8	7	9	10	10	0. 0	A1
215	169	110	75	50	35	33	33	33	36	43	55	71	82	2. 5	A2
274	230	175	128	81	51	48	50	51	58	73	98	128	149	5. 0	A3
322	300	242	181	112	67	63	68	71	80	104	143	186	218	7. 5	A4
360	360	268	216	130	72	70	77	82	95	131	182	243	283	10. 0	A5
410	450	369	284	172	98	95	101	106	122	165	228	301	353	12. 5	A6
474	469	402	320	190	104	100	110	116	137	190	267	354	417	15. 0	A7
548	488	469	374	222	121	117	128	136	160	221	311	413	486	17. 5	A8
622	553	531	423	249	135	130	143	151	180	249	352	467	550	20. 0	A9
290	262	248	209	122	78	77	80	74	92	127	180	247	288	10. 0	P1
0	2	6	9	7	6	5	5	5	6	5	5	8	8	0. 0	B1
108	116	106	85	55	35	32	32	32	33	38	51	68	78	2. 5	B2
221	194	176	143	83	53	49	52	52	55	73	98	133	154	5. 0	B3
309	273	250	200	127	79	73	74	77	93	109	151	194	229	7. 5	B4
261	250	257	212	130	66	63	70	74	89	123	177	237	279	10. 0	B5
352	331	334	275	164	89	89	93	97	116	159	229	291	350	12. 5	B6
421	395	396	322	190	101	95	103	109	132	184	265	354	418	15. 0	B7
482	453	456	371	217	113	107	118	126	151	212	305	409	483	17. 5	B8
555	520	521	423	249	130	124	136	143	172	241	349	467	552	20. 0	B9
267	236	221	181	112	72	70	73	70	80	106	152	196	232	8. 0	P2
0	4	6	8	10	10	11	12	13	13	13	14	15	17	0. 0	C1
67	82	90	70	51	36	40	42	43	42	52	67	80	90	2. 5	C2
230	166	167	139	98	75	69	72	74	76	94	119	144	165	5. 0	C3
241	230	235	190	122	85	86	90	90	98	123	160	200	230	7. 5	C4
299	300	301	239	158	123	132	135	126	126	157	196	245	265	10. 0	C5
430	405	372	298	186	120	125	131	130	145	185	245	315	360	12. 5	C6
512	470	438	347	215	138	137	144	145	162	210	290	374	431	15. 0	C7
565	540	500	395	240	150	149	160	162	183	242	330	430	502	17. 5	C8
600	583	557	440	263	160	159	170	173	200	270	370	490	568	20. 0	C9
253	219	199	163	113	73	72	73	73	77	89	133	152	184	6. 0	P3
0	7	14	9	7	12	13	14	14	18	17	18	19	19	0. 0	D1
130	79	87	74	57	41	40	38	38	41	51	66	84	94	2. 5	D2
202	145	151	128	90	61	56	58	58	62	83	110	143	161	5. 0	D3
270	210	215	175	120	71	70	73	74	86	112	152	198	229	7. 5	D4
350	280	283	230	150	90	89	96	95	107	142	196	254	292	10. 0	D5
405	330	340	275	174	102	95	105	106	128	174	240	310	364	12. 5	D6
460	389	401	324	204	115	110	123	125	147	200	280	372	433	15. 0	D7
460	403	436	360	223	120	117	127	134	160	225	318	421	494	17. 5	D8
460	420	471	390	241	128	120	136	145	174	250	355	471	550	20. 0	D9
190	160	140	100	64	54	54	59	60	50	70	90	120	140	4. 0	P4

Modelling:

Using the DEFINE function a data file (in this case TRIAL.DAT) can be set up with the required number of variables (here 15) and a systems file (here TRIAL.SYS) created and stored. The FINP function can then be used to input the data matrix which is stored on floppy disc.

Once the data file has been defined and stored on disc any combination of objects can be used to generate the model. Using the CLOAD function the combination of objects (in this case all the sets of standard solutions A1 to A9, B1 to B9, C1 to C9, and D1 to D9) from which the model will be generated can be set, labelled (given a class number) and the objects autoscaled.

Using the CPLS2 function a PLS model can be developed. Each principal component (C) is calculated in turn. If a value called the CSV/SD is < 0.95 then the PC is significant, and if it is > 1.0 then the PLS dimension predicts more noise than signal and is statistically insignificant.

The above procedure of extracting PLS components is continued until it is thought that the criteria will not hold at which time the correct number of PC's can be Stored.

The final step in the SIMCA procedure is to use the CLASSI function to fit objects to the SIMCA model and print out all the predicted values.

The PLS predicted values for Blankophor concentration were tabulated (TABLE 5.2) and the values corrected for the blank (zero predictions ie A1, B1, C1, D1). The error between the predicted values and the calculated standard values were calculated and these and the percentage errors are included in TABLE 5.2.

TABLE 5.2

PLS Predicted Blankophor Concentrations With Percentage Errors.

OBJECT NAME	BLANKOPHOR CONC/ppm	PLS PREDICTED CONC/ppm	CORRECTED CONC/ppm	ERROR	% ERROR
A1	0.00	-0.20	0.00	0.00	0.00
A2	2.50	2.29	2.49	-0.01	-0.40
A3	5.00	4.82	5.02	+0.02	+0.40
A4	7.50	7.46	7.66	+0.16	+2.13
A5	10.00	9.80	10.00	0.00	0.00
A6	12.25	12.64	12.84	+0.34	+2.64
A7	15.00	14.84	15.04	+0.04	+0.27
A8	17.50	17.34	17.54	+0.04	+0.23
A9	20.00	19.72	19.92	-0.08	-0.40
P1	10.00	9.80	10.00	0.00	0.00
B1	0.00	-0.21	0.00	0.00	0.00
B2	2.50	2.50	2.71	+0.21	+8.00
B3	5.00	5.18	5.38	+0.38	+7.60
B4	7.50	7.83	8.04	+0.53	+6.54
B5	10.00	9.86	10.07	+0.07	+0.05
B6	12.50	12.40	12.61	+0.11	+0.88
B7	15.00	15.01	15.22	+0.22	+1.40
B8	17.50	17.44	17.65	+0.15	+0.80
B9	20.00	19.97	20.18	+0.18	+0.80
P2	8.00	7.79	8.00	0.00	0.00
C1	0.00	-0.02	0.00	0.00	0.00
C2	2.50	2.73	2.75	+0.23	+8.80
C3	5.00	5.15	5.17	+0.17	+2.60
C4	7.50	7.79	7.81	+0.31	+3.73
C5	10.00	9.07	9.09	-0.91	-9.30
C6	12.50	12.56	12.58	+0.08	+0.48
C7	15.00	15.14	15.16	+0.14	+0.87
C8	17.50	17.72	17.74	+0.24	+1.26
C9	20.00	20.33	20.35	+0.35	+1.60
P3	6.00	6.00	6.02	+0.02	+0.33
D1	0.00	0.12	0.00	0.00	0.00
D2	2.50	2.67	2.55	+0.05	+2.00
D3	5.00	5.16	5.04	+0.04	+0.80
D4	7.50	7.66	7.54	+0.04	+0.53
D5	10.00	10.01	9.89	-0.11	-0.98
D6	12.50	12.66	12.54	+0.04	+0.40
D7	15.00	15.23	15.11	+0.11	+0.73
D8	17.50	17.56	17.44	-0.06	-0.34
D9	20.00	19.74	19.62	-0.38	-1.80
P4	4.00	4.16	4.04	+0.04	+1.00

Examination of Table 5.2 shows that the SIMCA model has predicted the four "unknown" Blankophor solutions very accurately. Solutions P1 and P2 were predicted exactly while solutions P3 and P4 had +0.33 and +1.0 percentage errors respectively.

The predicted values of the standard solutions used to set up the model are not quite as accurate as those predicted for the "unknown" solutions. With the exception of solutions B2, B3, B4, C2, and C5, whose errors were +8.0, +7.6, +6.54, +8.8 and -9.3% respectively, the errors are between -1.8 and +3.7%. This is more than acceptable for an analytical method and it was thus decided to undertake further biodegradation studies on Blankophor using SIMCA modelling to monitor the concentration.

Biodegradation Studies Using SIMCA Modelling.

Experiment 1.

Activated sludge ($200cm^3$) was added to each of six measuring cylinders ($2000cm^3$) and distilled water added up to the $1000cm^3$ mark. The solutions were continuously aerated and left to equilibrate for twenty four hours.

Blankophor (10ppm) was added to one of the cylinders and samples ($50cm^3$) taken from it periodically (T = 1, 24, 48, 96, and 120 hours). The samples were filtered; Whatman number 1, then Whatman number 44 ashless. Each time the test cylinder was sampled, filtered activated sludge solution was prepared from one of the blank cylinders and used to make up standard solutions of Blankophor (0 to 20ppm) (A1 to A9, B1 to B9, C1 to C9, D1 to D9, and E1 to E9).

Ultraviolet absorbance readings were measured for the above solutions at fourteen evenly spaced wave lengths from 210 to 240nm (Table 5.3).

TABLE 5.3 Ultraviolet Absorbance of Blankophor Solutions.

Wavelength/nm (Absorbance A x 10^{-3}														Conc.	Object
210	220	230	240	250	260	270	280	290	300	310	320	330	340	ppm	Name
0	8	3	6	6	7	8	8	8	8	8	7	8	10	0.0	A1
315	189	110	75	50	35	33	33	33	36	43	55	71	82	2.5	A2
384	255	175	128	82	51	48	50	51	58	73	98	128	149	5.0	A3
442	323	242	181	112	67	63	68	71	80	104	143	186	218	7.5	A4
403	375	268	216	130	72	70	77	82	95	131	182	243	283	10.0	A5
596	450	369	284	172	98	95	101	106	122	165	228	301	353	12.5	A6
474	419	402	320	190	104	100	110	116	137	190	267	354	417	15.0	A7
548	488	469	374	222	121	117	128	136	160	221	311	413	486	17.5	A8
622	553	531	423	249	135	130	143	151	180	249	352	467	550	20.0	A9
336	325	307	248	211	218	265	260	102	142	144	154	149	147	10.0	T1
0	2	3	4	5	5	5	6	7	7	8	15	17	17	0.0	B1
57	59	63	53	31	18	18	20	23	27	37	57	73	84	2.5	B2
135	129	131	104	61	34	34	38	40	49	67	101	131	151	5.0	B3
207	192	192	154	90	50	49	55	58	70	98	145	189	220	7.5	B4
275	258	257	204	118	67	68	75	77	92	127	188	245	284	10.0	B5
395	347	330	262	150	83	81	89	95	115	159	234	307	358	12.5	B6
465	413	394	309	179	100	98	107	113	136	190	277	364	425	15.0	B7
557	489	461	361	208	114	113	124	130	157	220	320	422	493	17.5	B8
630	555	526	412	237	131	128	141	149	178	250	364	479	561	20.0	B9
1362	1355	118	162	134	147	196	194	140	115	122	136	134	113	??	T24
0	3	6	8	9	10	11	12	12	12	12	14	15	16	0.0	C1
76	84	91	77	56	44	44	46	46	47	55	68	82	92	2.5	C2
170	163	161	132	90	65	66	70	68	72	87	113	140	158	5.0	C3
244	234	231	188	124	85	85	90	88	96	120	160	201	229	7.5	C4
291	302	301	236	155	116	128	131	118	120	147	192	236	260	10.0	C5
433	403	374	296	187	122	124	130	129	142	182	246	313	360	12.5	C6
510	476	437	345	213	134	134	143	144	160	210	287	371	429	15.0	C7
562	539	501	396	241	149	148	158	160	181	240	331	430	499	17.5	C8
597	588	556	441	265	160	158	170	174	199	267	372	486	565	20.0	C9
1310	1310	93	118	95	108	151	148	100	79	86	100	97	79	??	T48
0	8	18	25	29	31	32	33	34	35	36	38	41	42	0.0	D1
168	142	112	86	73	74	87	87	73	68	71	77	79	75	2.5	D2
573	542	240	133	101	103	130	130	103	91	95	105	106	97	5.0	D3
540	474	254	158	118	124	163	163	125	109	117	130	133	121	7.5	D4
361	371	313	213	157	164	216	216	163	137	146	163	165	148	10.0	D5
445	460	383	257	184	189	252	252	188	160	172	197	203	184	12.5	D6
493	525	449	305	216	218	289	289	216	183	199	229	240	221	15.0	D7
560	611	522	351	246	249	332	332	246	207	226	260	272	249	17.5	D8
617	690	594	392	277	287	386	385	280	230	248	286	293	260	20.0	D9
437	371	321	239	178	157	169	164	137	127	137	164	181	184	??	T96
0	11	19	25	29	31	33	34	35	36	37	38	41	42	0.0	E1
238	161	111	91	71	58	58	59	59	63	72	85	100	111	2.5	E2
443	283	178	137	97	73	73	76	76	82	99	125	152	172	5.0	E3
505	338	233	185	125	86	86	91	93	103	128	167	211	242	7.5	E4
346	291	268	221	143	92	95	102	103	117	152	204	260	297	10.0	E5
457	390	356	291	187	120	120	128	130	147	190	256	327	378	12.5	E6
548	471	427	346	219	136	134	143	148	169	221	301	389	451	15.0	E7
522	494	484	396	247	151	149	160	166	189	251	344	445	516	17.5	E8
586	552	538	439	272	164	162	175	181	208	277	384	497	578	20.0	E9
502	364	262	169	120	112	135	132	101	88	95	106	114	109	??	T120

A SIMCA model was generated using the five sets of standard Blankophor solutions. A print out was obtained of the predicted Blankophor concentrations for all the solutions and the values tabulated (Table 5.4).

Examination of Table 5.4 shows that the Simca model generated using all the sets of standards predicts the concentrations of the standard Blankophor solutions accurately.

The concentration of Blankophor in the test cylinder fell from 10.42ppm to 6.48ppm over the test period of five days, indicating that some change: biodegradation and/or trans/cis isomerisation (adsorption onto the solid material of the activated sludge is unlikely as previously indicated in Chapter 2) had occurred to the Blankophor.

TABLE 5.4 PLS Predicted Blankophor Concentrations:

OBJECT NAME	BLANKOPHOR CONC/PPM	BLANK CORRECTED PLS PREDICTED CONC/PPM	OBJECT NAME	BLANKOPHOR CONC/PPM	BLANK CORRECTED PLS PREDICTED CONC/PPM
A1	0.0	00.00	C6	12.5	12.52
A2	2.5	2.63	C7	15.0	15.14
A3	5.0	5.09	C8	17.5	17.59
A4	7.5	7.60	C9	20.0	19.96
A5	10.0	10.23	T48	??	7.22
A6	12.5	12.81	D1	0.0	0.00
A7	15.0	15.10	D2	2.5	2.58
A8	17.5	17.63	D3	5.0	5.04
A9	20.0	20.00	D4	7.5	7.36
T1	10.0	10.42	D5	10.0	10.04
B1	0.0	00.00	D6	12.5	12.63
B2	2.5	2.50	D7	15.0	15.05
B3	5.0	5.00	D8	17.5	17.58
B4	7.5	7.50	D9	20.0	19.85
B5	10.0	10.06	T96	??	6.80
B6	12.5	12.52	E1	0.0	0.00
B7	15.0	14.88	E2	2.5	2.44
B8	17.5	17.70	E3	5.0	4.85
B9	20.0	20.00	E4	7.5	7.47
T24	??	8.58	E5	10.0	9.88
C1	0.0	0.00	E6	12.5	12.48
C2	2.5	2.42	E7	15.0	14.96
C3	5.0	5.02	E8	17.5	17.45
C4	7.5	7.47	E9	20.0	19.69
C5	10.0	10.0	T120	??	6.48

Experiment 2.

To verify the results from the previous experiment, and to see if the results were reproducible between different batches of activated sludge a similar experiment was set up.

In this case eight blank, and two test cylinders were set up, as before, with samples being taken at T = 48, 96, 120 and 144 hours and treated as before.

The ultraviolet absorbance readings are recorded in Table 5.5 and the PLS predicted Blankophor concentrations (using all the sets of standards as the model) in Table 5.6.

TABLE 5.5 Ultraviolet Absorbance Readings of Blankophor Solutions.

Wavelength/nm (Absorbance Å x 10^{-3}														Conc.	Object
210	220	230	240	250	260	270	280	290	300	310	320	330	340	ppm	Name
0	0	22	30	26	24	22	20	19	17	16	14	15	15	0.0	A1
47	47	46	102	92	75	69	64	58	56	61	68	81	90	2.5	A2
38	36	64	105	75	49	49	50	48	53	69	93	120	138	5.0	A3
54	54	115	154	97	57	55	58	58	67	92	131	174	203	7.5	A4
82	83	144	204	134	83	82	86	86	96	128	180	236	273	10.0	A5
108	107	190	257	164	99	96	101	101	115	155	221	290	338	12.5	A6
132	134	240	312	201	122	120	125	122	137	184	262	345	401	15.0	A7
141	140	252	342	213	124	128	134	131	150	206	295	390	455	17.5	A8
130	132	239	352	211	110	113	126	126	149	216	321	432	508	20.0	A9
62	60	94	158	109	60	62	70	72	82	115	150	198	227	??	AT48
0	0	10	22	25	28	22	21	21	18	17	15	17	18	0.0	B1
46	43	67	99	84	66	63	64	64	65	67	80	93	99	2.5	B2
38	37	41	99	82	54	52	53	52	58	73	99	128	148	5.0	B3
56	54	84	148	100	58	59	66	69	79	105	145	188	217	7.5	B4
74	72	114	188	121	66	68	78	82	96	130	184	241	281	10.0	B5
83	85	142	223	136	69	72	83	87	105	148	215	285	334	12.5	B6
116	115	197	293	184	101	100	110	112	131	181	262	346	406	15.0	B7
122	121	200	317	195	101	101	112	117	140	200	295	393	464	17.5	B8
127	123	205	334	199	94	95	108	116	147	216	525	440	521	20.0	B9
62	59	91	150	110	59	60	69	70	82	116	152	200	228	??	BT48
0	1	18	31	29	26	25	25	23	22	20	19	21	21	0.0	C1
24	23	55	75	62	50	45	42	39	40	46	58	73	83	2.5	C2
44	42	84	120	84	58	56	56	54	58	73	98	127	146	5.0	C3
66	63	152	182	110	68	66	70	68	76	99	138	181	209	7.5	C4
98	98	184	241	154	98	98	105	100	109	142	193	248	285	10.0	C5
120	124	232	297	184	117	115	121	120	133	174	239	305	353	12.5	C6
160	162	292	375	237	154	149	153	146	160	207	284	365	420	15.0	C7
140	142	253	359	219	127	125	133	131	151	207	301	397	464	17.5	C8

TABLE 5.5 CONT

Wavelength/nm (Absorbance Ā x 10^{-3})														CONC.	OBJECT
210	220	230	240	250	260	270	280	290	300	310	320	330	340	PPM	NAME
169	171	294	425	262	154	150	160	156	176	240	343	451	527	20.0	C9
40	30	46	106	70	34	34	41	47	61	82	130	175	200	??	AT96
0	0	8	26	25	24	22	21	20	19	17	21	22	23	0.0	D1
42	38	54	92	77	61	58	57	52	51	58	72	86	95	2.5	D2
40	38	54	102	79	54	53	57	56	62	76	106	134	154	5.0	D3
53	54	88	146	98	59	59	64	65	74	98	141	184	214	7.5	D4
66	65	89	167	110	60	62	69	71	85	119	176	232	273	10.0	D5
104	102	170	253	162	94	94	103	104	120	162	231	300	349	12.5	D6
127	125	191	296	193	113	114	124	127	144	194	276	359	417	15.0	D7
120	118	188	300	186	100	101	114	120	142	201	297	393	461	17.5	D8
90	88	106	253	158	65	70	87	100	129	200	311	425	506	20.0	D9
39	29	46	104	69	33	34	40	46	60	79	129	174	201	??	BT96
0	-10	-1	11	11	11	11	11	11	12	12	19	21	22	0.0	E1
24	6	43	62	36	23	24	26	25	29	37	58	74	83	2.5	E2
42	35	75	114	73	47	48	51	50	56	73	106	134	153	5.0	E3
50	45	97	148	86	47	48	55	55	66	92	142	185	214	7.5	E4
68	64	132	195	109	58	62	70	72	86	121	185	242	279	10.0	E5
76	70	145	222	123	61	64	75	79	97	139	217	288	336	12.5	E6
40	38	113	195	82	11	18	34	44	70	126	221	309	327	15.0	E7
64	62	123	232	114	33	41	59	69	98	162	268	368	439	17.5	E8
138	136	126	312	216	123	129	143	144	169	235	349	458	532	20.0	E9
27	21	34	20	60	28	28	31	38	52	69	114	161	179	??	AT120
0	0	4	20	21	20	16	19	19	20	19	28	29	31	0.0	F1
24	19	24	58	55	41	40	41	40	42	51	75	90	99	2.5	F2
41	40	62	99	76	49	49	52	51	57	74	112	141	161	5.0	F3
86	84	85	122	182	131	89	94	93	102	126	175	218	246	7.5	F4
94	88	126	202	142	88	89	97	96	108	143	207	264	301	10.0	F5
91	88	132	225	146	80	81	90	93	109	152	232	304	353	12.5	F6
72	68	101	213	121	50	55	68	73	92	144	239	323	381	15.0	F7
75	69	121	230	130	50	57	73	77	101	162	271	367	432	17.5	F8
90	84	140	270	149	52	55	74	82	110	181	310	423	502	20.0	F9
27	20	33	96	60	28	28	31	37	51	69	114	161	179	??	BT120
0	-10	6	21	16	16	14	12	11	10	9	16	17	18	0.0	G1
18	9	32	62	42	30	28	29	28	30	36	57	71	81	2.5	G2
42	32	67	107	71	47	46	48	48	54	69	104	132	152	5.0	G3
45	32	65	117	76	40	40	46	49	58	83	130	173	204	7.5	G4
84	78	129	195	129	78	77	82	83	95	128	187	244	283	10.0	G5
95	82	139	215	140	80	82	90	92	108	150	223	293	342	12.5	G6
116	110	167	265	172	98	97	106	109	127	177	264	347	405	15.0	G7
58	54	116	210	115	38	44	57	60	82	142	243	339	405	17.5	G8
102	102	191	299	179	84	84	95	100	124	192	307	419	497	20.0	G9
40	39	62	99	75	48	48	52	51	57	74	112	141	161	??	AT144
0	0	6	23	24	23	22	21	20	20	19	26	28	28	0.0	H1
11	2	2	37	31	23	21	24	25	28	36	56	72	84	2.5	H2
48	43	43	97	79	57	56	59	60	65	82	116	145	164	5.0	H3
40	32	47	107	70	35	35	42	47	60	83	131	175	206	7.5	H4
70	60	97	167	105	55	56	64	68	81	116	176	233	273	10.0	H5
134	131	186	280	194	129	128	135	136	150	191	261	329	377	12.5	H6
145	142	213	316	212	134	133	140	143	160	210	293	375	434	15.0	H7
90	86	139	252	150	66	68	80	89	114	175	275	373	444	17.5	H8
127	122	203	334	196	97	101	117	124	151	220	332	443	521	20.0	H9
40	40	61	100	75	48	47	52	51	57	74	110	140	160	??	BT144

TABLE 5.6 PLS Predicted Blankophor Concentrations:

OBJECT NAME	BLANKOPHOR CONC/PPM	BLANK CORRECTED PLS PREDICTED CONC/PPM	OBJECT NAME	BLANKOPHOR CONC/PPM	BLANK CORRECTED PLS PREDICTED CONC/PPM
A1	0.0	0.00	E1	0.0	0.00
A2	2.5	2.52	E2	2.5	2.46
A3	5.0	4.94	E3	5.0	5.10
A4	7.5	7.59	E4	7.5	7.72
A5	10.0	9.92	E5	10.0	10.19
A6	12.5	12.37	E6	12.5	12.53
A7	15.0	15.05	E7	15.0	14.92
A8	17.5	17.38	E8	17.5	17.44
A9	20.0	20.06	E9	20.0	19.82
AT48	??	8.70	AT120	??	6.91
B1	0.0	0.00	F1	0.0	0.00
B2	2.5	2.27	F2	2.5	3.07
B3	5.0	5.20	F3	5.0	5.21
B4	7.5	7.62	F4	7.5	7.54
B5	10.0	10.00	F5	10.0	10.54
B6	12.5	12.41	F6	12.5	12.62
B7	15.0	15.28	F7	15.0	15.12
B8	17.5	17.80	F8	17.5	17.54
B9	20.0	19.97	F9	20.0	20.16
BT48	??	9.00	BT120	??	6.81
C1	0.0	0.00	G1	0.0	0.00
C2	2.5	2.58	G2	2.5	2.36
C3	5.0	4.88	G3	5.0	4.99
C4	7.5	7.40	G4	7.5	7.46
C5	10.0	10.05	G5	10.0	10.14
C6	12.5	12.33	G6	12.5	12.60
C7	15.0	14.98	G7	15.0	14.94
C8	17.5	17.43	G8	17.5	17.18
C9	20.0	19.80	G9	20.0	20.28
AT96	??	7.12	AT144	??	5.59
D1	0.0	0.00	H1	0.0	0.00
D2	2.5	2.68	H2	2.5	2.45
D3	5.0	5.02	H3	5.0	4.80
D4	7.5	7.58	H4	7.5	7.50
D5	10.0	9.96	H5	10.0	9.78
D6	12.5	12.73	H6	12.5	12.42
D7	15.0	15.14	H7	15.0	15.01
D8	17.5	17.22	H8	17.5	17.36
D9	20.0	20.02	H9	20.0	19.80
BT96	??	7.19	BT144	??	5.44

It can be seen from TABLE 5.5 that the absorbance readings are different to those obtained in experiment 1. This is almost certainly due to the difference in the composition of the two activated sludge batches used.

Examination of TABLE 5.6, however, shows that the PLS predicted Blankophor concentrations for the sets of standards are very similar. Over the test period of six days the concentration of Blankophor falls from 10ppm to approximately 5.5ppm, a similar pattern to that seen in experiment 1. These results thus confirm the reproducibility of results between different batches of activated sludge and the accuracy of the SIMCA model predictions.

Two further experiments were carried out, with fresh batches of activated sludge, using the same procedure as in experiment 1. The results further confirmed that the Blankophor concentration fell from 10ppm to approximately 5.5ppm (10.2ppm to 5.7ppm and 10.0 to 5.4ppm in the above experiments) over six days.

Although the "fit" of the SIMCA PLS model predictions for Blancophor concentrations was good for both the test and standard solutions, it could be seen, by visual inspection, that the ultraviolet spectrum of the test solution changed during the test period.

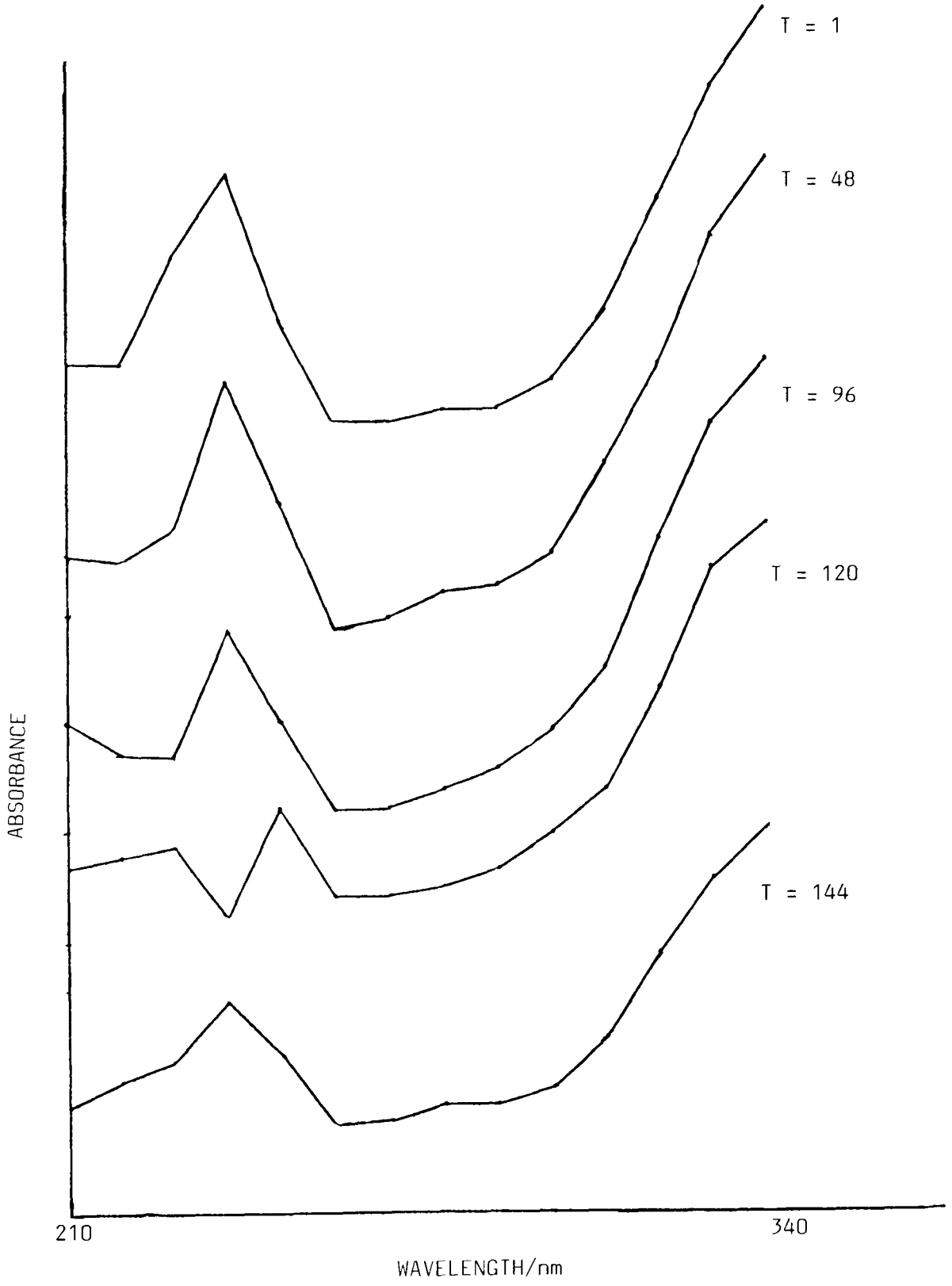
Graphs of absorbance versus wavelength were constructed (FIG. 5.1) for the test solutions in experiment 2. Examination of these graphs show that there is a change in the test solution spectrum in the region 210 to 260nm.

If the Blankophor had merely been adsorbed onto the solid material of the activated sludge solution there should have been no change in its ultraviolet spectrum.

If the change in the ultraviolet spectrum was due to metabolism of some of the compounds in the activated sludge solutions, the spectra of the standard solutions would also have changed, but this was not the case.

The drop in Blankophor concentration coupled with the changes in the ultraviolet spectrum indicate that some 45% of the Blankophor had either

Fig. 5.1 Ultraviolet Spectra of the Test Solutions from Experiment 1.



been metabolised, isomerised, or a combination of both.

It was thus decided to examine methods to try to separate the Blankophor and the metabolites and/or isomers from the activated sludge solution, with a view to then separating them into their component compounds and identifying them.

The Blankophor is in the sodium form and it should be possible to ionise it at low pH. Examination of a 3-dimensional computer model (created using the CHEM-X facility on the VAX mainframe computer) of the Blankophor indicated that there was no steric reasons why it could not be ion-paired. Indeed most of the nitrogen molecules had an associated negative charge.

Ion-pairing was thus considered as a method of separation. Before any attempts were tried to extract the six day biodegradation solutions, attempts were made to separate the Blankophor from the filtered activated sludge solution medium.

Because of the similarities between the ultraviolet spectra of the six day biodegradation solution and that of the Blankophor solution it would seem likely that any technique that could extract the Blankophor would also extract the metabolites and/or isomers.

Two ion-pairing reagents were examined; tetraphenyl arsonium chloride (a well known ion-pairing reagent) and poly[oxyethylene(dimethylimino)-ethylene(diethylimino)-ethylene] dichloride (WSCP) (a newer polymeric material supplied by Buckman Laboratories Inc.).

SOLVENT EXTRACTION OF BLANKOPHOR USING ION-PAIRING REAGENTS.

Solvent Extraction of Blankophor Using Ion-Pairing Reagents.

Introduction:[40, 41]

Solvent extraction, sometimes called liquid-liquid extraction, involves the selective transfer of a substance from one phase to another. Usually an aqueous solution of the sample is extracted with an immiscible organic solvent.

The extraction of a solute in this manner is governed by the Nernst partition or distribution which states that at equilibrium, a given solute will always be distributed between two essentially immiscible liquids in the same proportions. Thus for solute A distributing between an aqueous and an organic solvent,

$$[A]_o/[A]_{aq} = K_D$$

where square brackets denote concentrations (strictly activities) and K_D is known as the equilibrium distribution or partition coefficient which is independent of total solute concentration. The value of K_D is a reflection of the relative solubilities of the solute in the two phases. In many practical situations solute A may dissociate, polymerise or form complexes with some other component of the sample or interact with one of the solvents. Under these circumstances the value of K_D does not reflect the overall distribution of the solute between the two phases as it refers only to the distributing species. The extraction process is therefor better discussed in terms of the distribution ratio D where

$$D = (C_a)_o/(C_A)_{aq}$$

where (C_A) represents the total concentration of all forms of solute A. Considerable variation in the experimental value of D can be achieved by altering solution conditions so that solvent extraction is a very versatile technique.

The basic requirement for a solute to be extractable from an aqueous

solution is that it should be uncharged, or can form part of an uncharged ionic aggregate (ion-pair). Charge neutrality reduces electrostatic interactions between the solute and water and hence lowers its aqueous solubility. Extraction into a less polar organic solvent is facilitated if the species is not hydrated, or if the co-ordinated water is easily displaced by hydrophobic co-ordinating groups such as bulky organic molecules. Salting out agents are often used to increase the distribution ratio. These are electrolytes, such as di- and tri-valent nitrates, with a pronounced tendency to hydration. They bind large numbers of water molecules thereby lowering the dielectric constant of the solution and favouring ion-association.

Earlier in this investigation, (Page 45-51) attempts were made, using various solvents, to extract Blankophor from aqueous solution but with no success. The Blankophor was in the sodium form and, if ionised, the charged species would inhibit extraction.

As discussed earlier in this investigation (Page 52-69) the many nitrogens in the Blankophor carry residual negative charges; again this may have inhibited any extraction. Working at high pH would probably eliminate any ionisation of the Blankophor molecule but this would not solve the problem of the residual charges on the nitrogens.

It was thought possible that working at low pH (2.0), using an ion pairing reagent in conjunction with a salting out agent, that the above problems could be overcome.

Two ion-pairing reagents:- tetraphenylarsonium chloride and a newer imino polymer, poly[oxyethylene(dimethylimino)-ethylene(diethylimino)-ethylene]dichloride, (WSCP)(supplied by Buckman Laboratories Inc.) were examined to see if they could be used to extract Blankophor, and or any

metabolites, from filtered activated sludge solution

In all the following solvent extractions the pH of the aqueous solutions were adjusted to 2.0 (2M HCl solution) prior to continuous extraction.

(a) Tetraphenylarsonium Chloride (TPAC):

Before undertaking any extractions from the filtered activated sludge solution Blankophor and TPAC were examined to determine their solubility in the ethyl acetate.

Infra-red (I.R.) spectra were obtained for the following:

Ethyl acetate (liq.) (Fig. 6.1).

Blankophor in ethyl acetate (approx. 10% sol.) (Fig. 6.2).

TPAC in ethyl acetate (approx. 10% sol.) (Fig. 6.3).

Blankophor/TPAC in ethyl acetate [TPAC, 1g, was dissolved in Blankophor solution ($10cm^3$, 10%) and stirred for one hour (magnetic stirrer) when it was mixed with ethyl acetate, $90cm^3$] (Fig 6.4)

Blankophor solid (KBr disc) (Fig 6.5).

TPAC solid (KBr disc) (Fig 6.6).

Comparison of Figs. 6.1 to 6.6 indicates that Blankophor and TPAC are not soluble in ethyl acetate.

Examination of Fig. 6.4 indicates that Blankophor/TPAC ion pair is also insoluble in ethyl acetate. However it should be noted that stirring the two compounds for one hour at room temperature may not have been sufficient to produce ion-pairing and thus Fig. 6.4 may indicate the absence of ion-pairing rather than the insolubility of the ion-pair.

TPAC (1g) was dissolved in Blankophor solution ($100cm^3$, 10ppm) in distilled water and sodium acetate (15g) added as a salting out agent. Ethyl acetate ($200cm^3$) was added and the mixture heated under reflux conditions for seven days. The organic layer was separated, dried (magnesium sulphate) and an I.R. spectrum obtained. (Fig. 6.7) For ease

of comparison this spectrum was superimposed on a spectrum of ethyl acetate. (Fig. 6.8)

Examination of Fig. 6.8 shows quite clearly that extraction has not been successful. The two spectra are virtually identical indicating that the Blankophor/TPAC ion-pair, if formed, can not be extracted using this method.

The above extraction was repeated using Blankophor dissolved in filtered activated sludge solution, rather than distilled water, to see if any of the interfering species could be ion-paired and extracted, but this again proved to be unsuccessful.

Various solvents (isobutyl alcohol, acetylacetone, and tetrachloro methane) were substituted for ethyl acetate and extractions carried out as previously. With all the solvents examined there was no extraction into the organic phase.

(b) Poly [oxyethylene (dimethylimino)-ethylene (diethylimino)-ethylene] dichloride (WSCP).

Before undertaking any extractions from the filtered activated sludge water, Blankophor and WSCP were examined to see how soluble they were in both distilled water and ethyl acetate.

Ultra-violet (U.V.) spectra were obtained for the following:

Ethyl acetate (liq.) (Fig. 6.9).

Blankophor solution (20ppm) in ethyl acetate (Fig. 6.10).

WSCP solution (800ppm) in ethyl acetate (Fig. 6.11).

Blankophor/WSCP aqueous solution (20ppm, 800ppm) Fig. 6.12).

Blankophor/WSCP in ethyl acetate [Blankophor and WSCP , 20ppm and 800ppm respectively were added to ethyl acetate and the solution stirred for

one hour] (Fig. 6.13).

Blankophor in distilled water (20ppm) (Fig. 6.14).

WSCP in distilled water (800ppm) (Fig. 6.15).

Comparison of Figs. 6.9 to 6.15 indicates that Blankophor and WSCP are not soluble in ethyl acetate.

Examination of Fig. 6.13 indicates that either the Blankophor/WSCP ion-pair is insoluble in ethyl acetate, or the ion-pair has not been formed. WSCP $1cm^3$) was dissolved in Blankophor solution ($50cm^3$, 10ppm) in distilled water and sodium acetate (5g) added as a salting out agent. Ethyl acetate was added ($100cm^3$) and the mixture heated under reflux conditions for seven days. The organic layer was separated, dried (magnesium sulphate) and an I.R. spectra obtained. (Fig. 6.16)

Comparing Fig. 6.16 with Fig. 6.1 (I.R. spectrum of ethyl acetate) indicates that no extraction has occurred.

The above extraction was repeated replacing the distilled water with filtered activated sludge solution, to see if any of the interfering species could be ion-paired and extracted but again with no success.

Various solvents (isobutyl alcohol, acetylacetone and tetrachloro methane) were substituted for ethyl acetate and extractions carried out as above. I.R. spectra were obtained for all the dried extracted organic phases and for the pure solvents. (Figs. 6.17, 6.18, 6.19, 6.20, 6.21, and 6.22) Examination of these spectra shows that no extraction had occurred.

Extraction of Blankophor may not have occurred because the pH may not have been low enough to cause ionisation. Also the salting out agent may not have been strong enough to replace the hydrating water molecules at the charged nitrogen sites in the Blankophor molecule.

Figs. 6.1 to 6.2 INFRA-RED SPECTRA

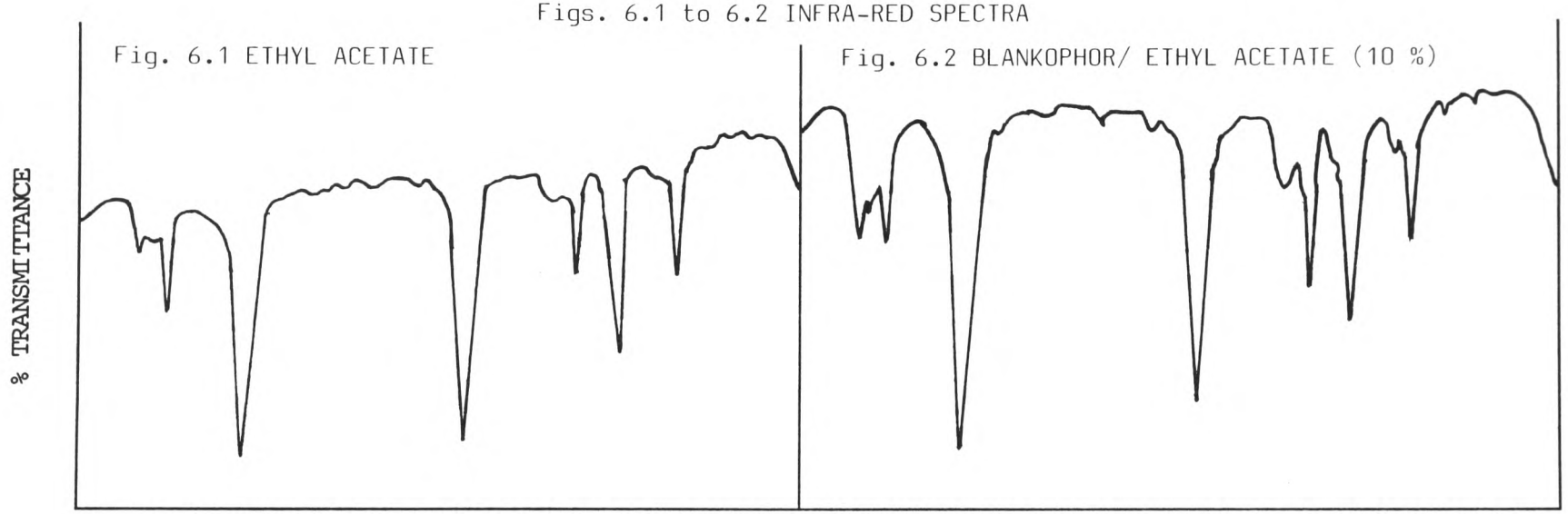


Fig. 6.1 ETHYL ACETATE

Fig. 6.2 BLANKOPHOR/ ETHYL ACETATE (10 %)

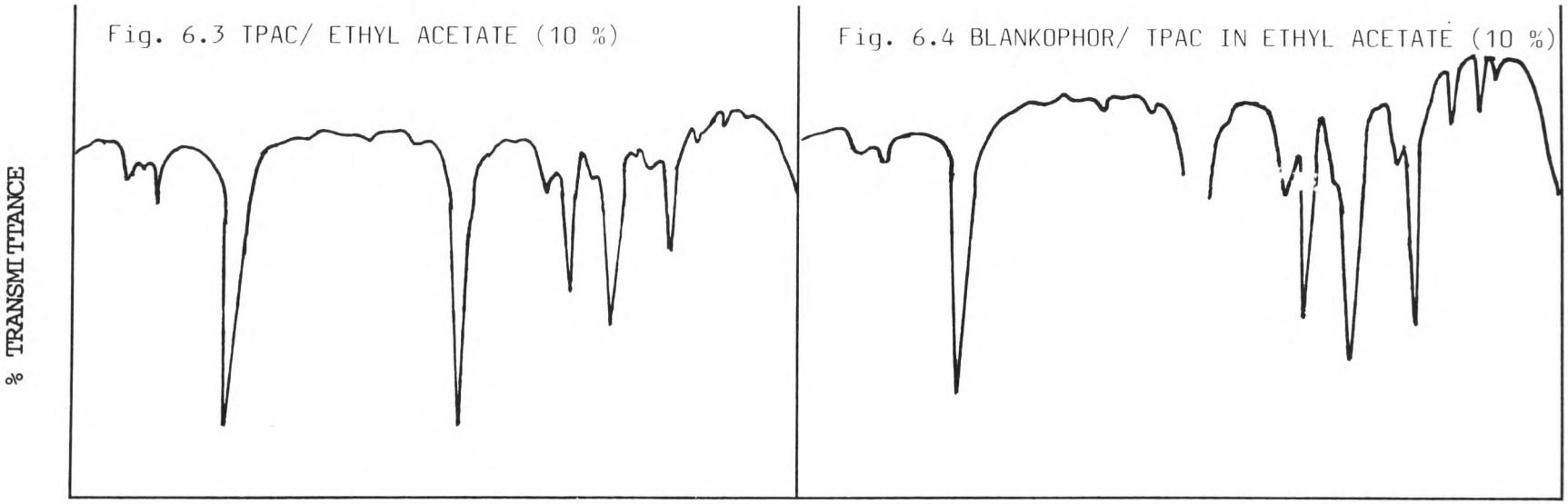
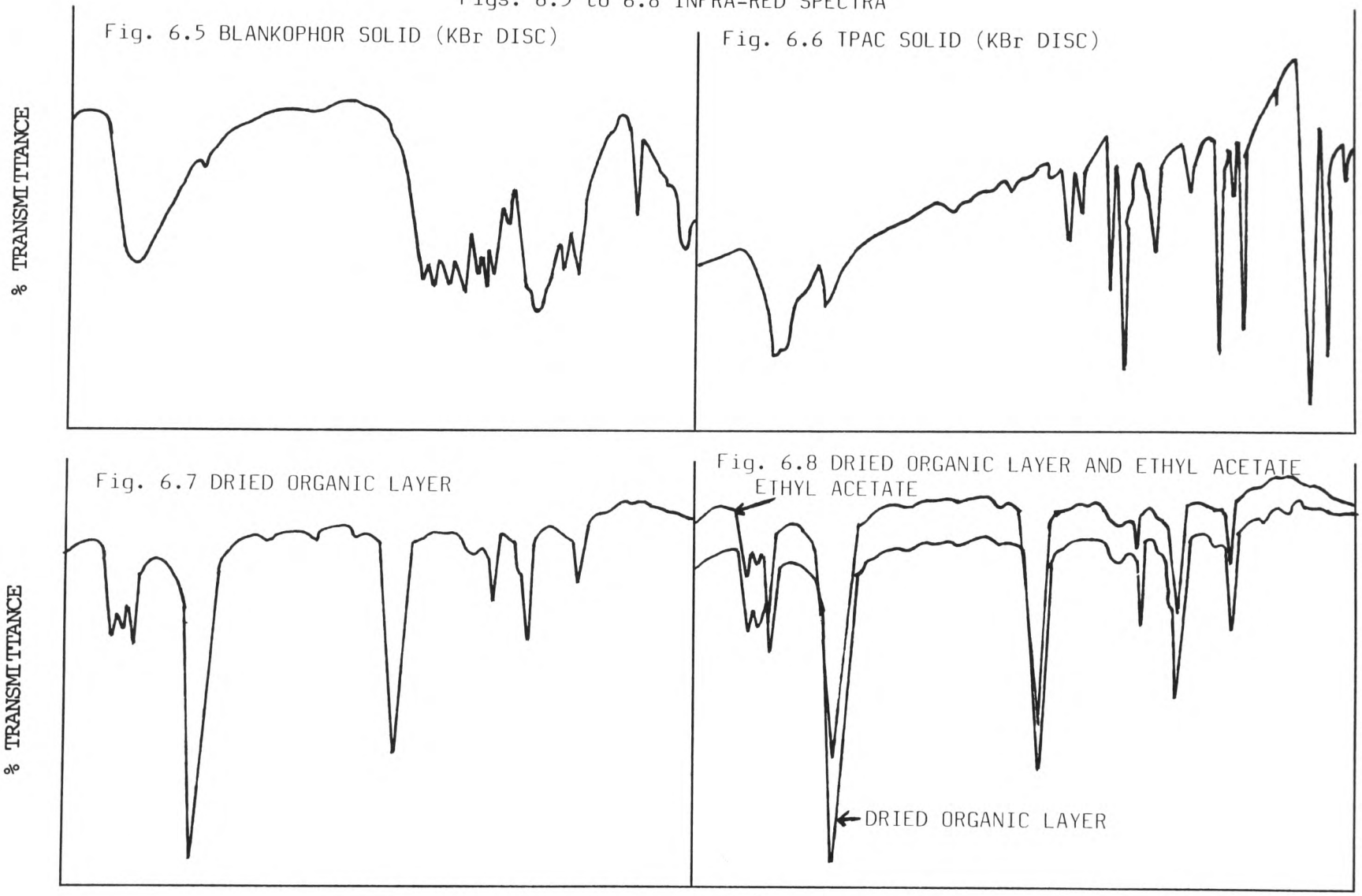


Fig. 6.3 TPAC/ ETHYL ACETATE (10 %)

Fig. 6.4 BLANKOPHOR/ TPAC IN ETHYL ACETATE (10 %)

Figs. 6.5 to 6.8 INFRA-RED SPECTRA



Figs. 6.9 to 6.12 ULTRAVIOLET SPECTRA.

Fig 6.9 ETHYL ACETATE

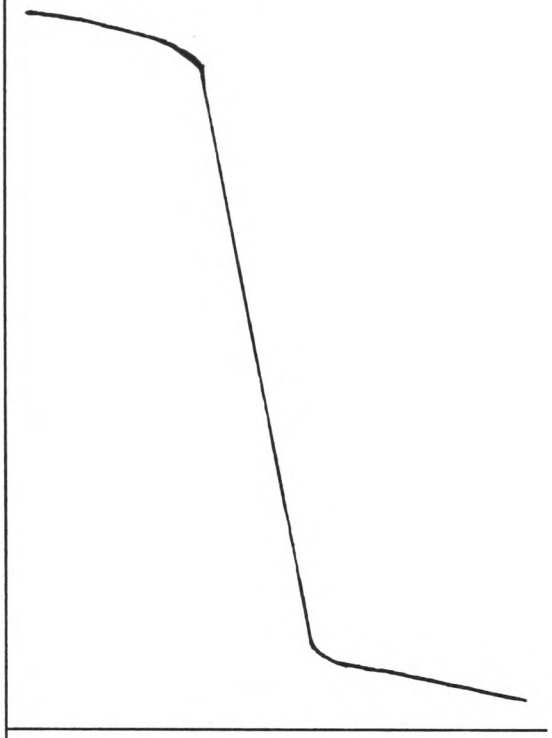


Fig 6.10 BLANKOPHOR IN ETHYL ACETATE (20ppm)

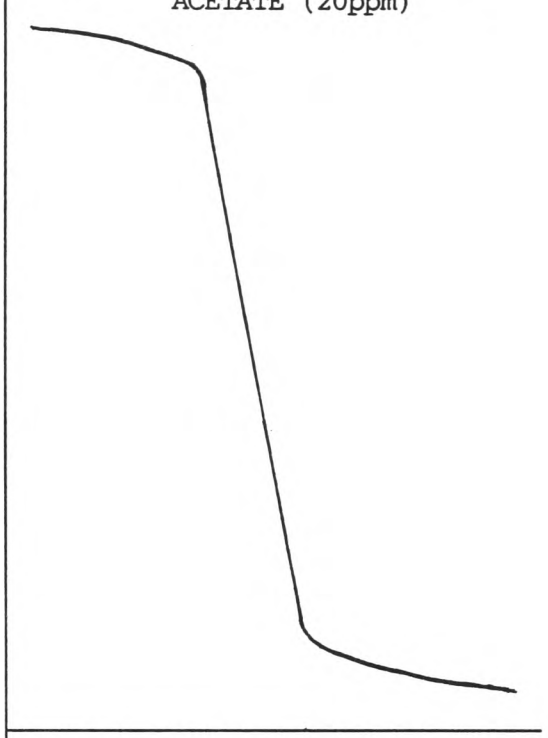


Fig 6.11 WSCP IN ETHYL ACETATE (800ppm)

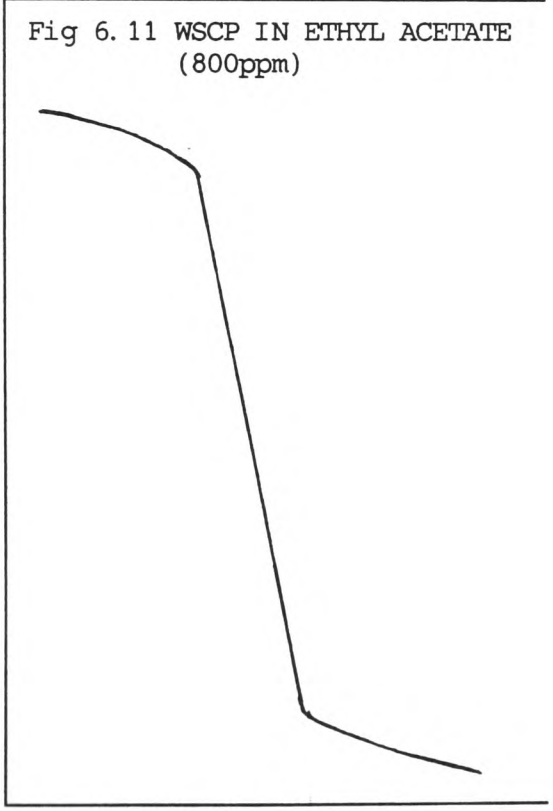
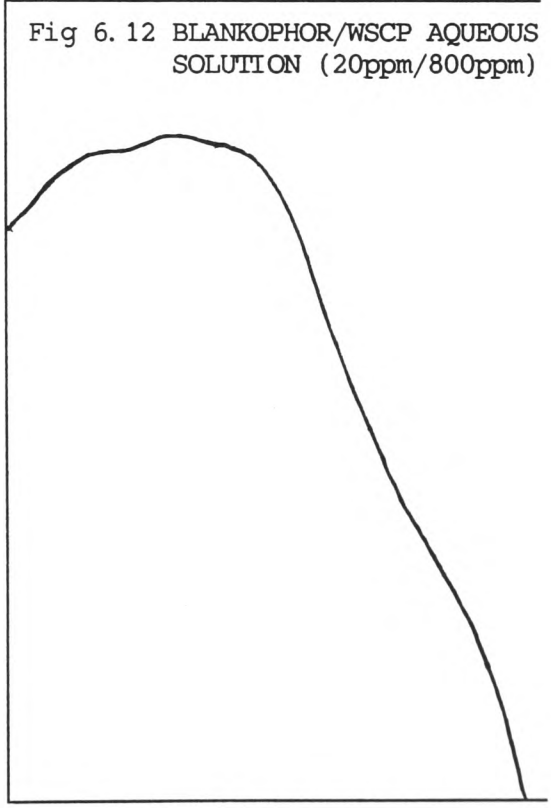


Fig 6.12 BLANKOPHOR/WSCP AQUEOUS SOLUTION (20ppm/800ppm)



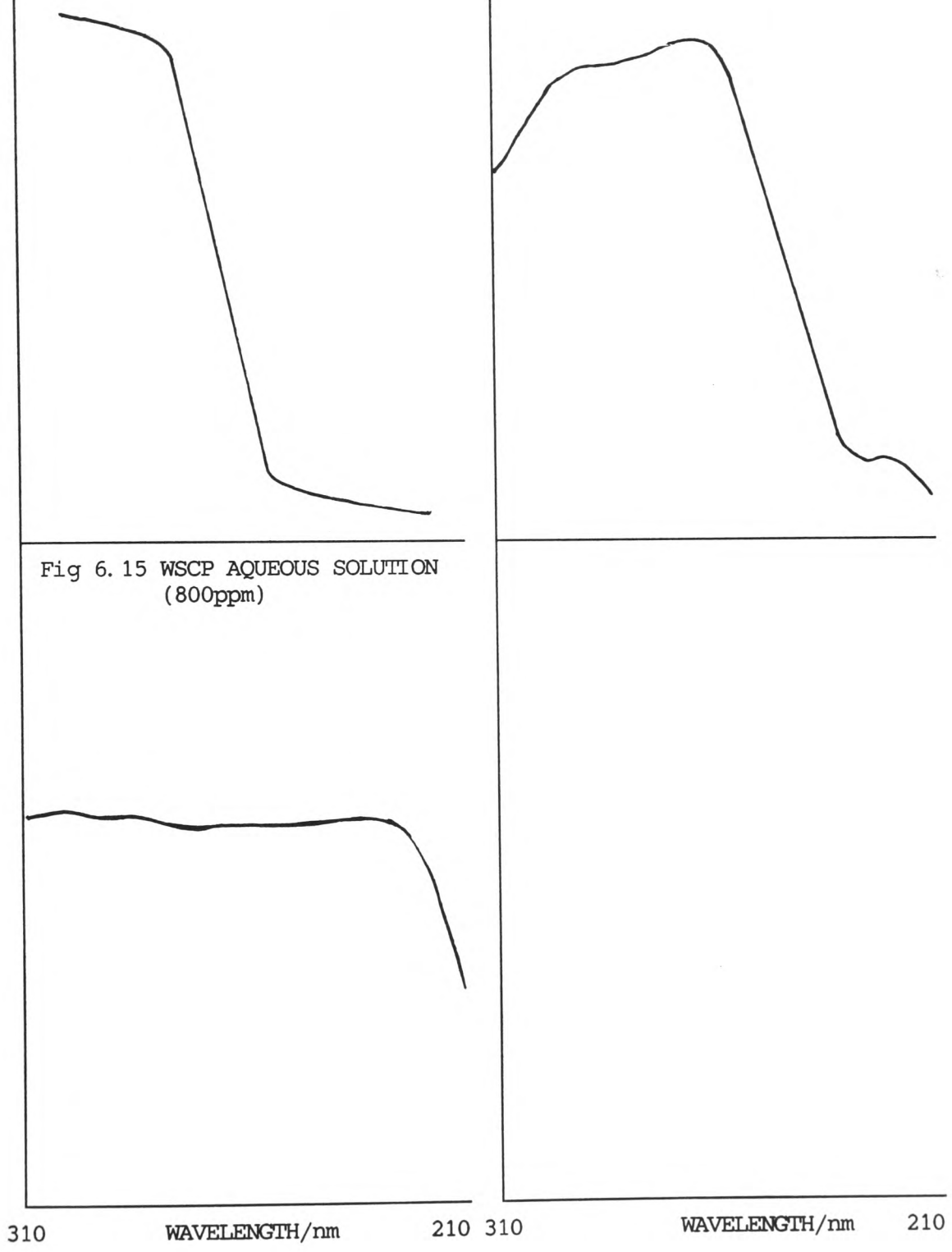
310 WAVELENGTH/nm 210 310 WAVELENGTH/nm 210

Figs 6.13 to 6.15 ULTRAVIOLET SPECTRA

Fig 6.13 BLANKOPHOR/WSCP IN ETHYL ACETATE (20ppm/800ppm)

Fig 6.14 BLANKOPHOR AQUEOUS SOLUTION (20ppm)

Fig 6.15 WSCP AQUEOUS SOLUTION (800ppm)



Figs. 6.16 to 6.19 INFRA-RED SPECTRA

Fig. 6.16 DRIED ORGANIC LAYER

Fig. 6.17 ISO-BUTYL ALCOHOL

Fig. 6.18 DRIED ORGANIC LAYER

Fig. 6.19 ACETYLACETONE

Fig. 6.20 DRIED ORGANIC LAYER

Fig. 6.22 DRIED ORGANIC LAYER

Figs. 6.20 to 6.22 INFRA-RED SPECTRA

Fig. 6.21 TETRACHLORO METHANE

EXTRACTION OF BLANKOPHOR USING THIN LAYER CHROMATOGRAPHY.

Extraction of Blankophor Using Thin Layer Chromatography. (TLC)

Introduction.

Chromatography has been defined as primarily a separation process which is used for the separation of essentially molecular mixtures. It depends upon the redistribution of the molecules of the mixture between two or more phases. Distribution takes place between a 'stationary' sorbed 'liquid' phase and a mobile fluid in intimate contact with it.

The technique of TLC uses an adsorbent coated on a glass plate as the stationary phase and development of the chromatogram takes place as the mobile phase percolates through the adsorbent.

In TLC a variety of coating materials are available, although silica gel was used more often than other materials and will be used in this investigation.

Thin layers of silica gel can be made by spreading an aqueous slurry of silica gel powder using one of the commercially available applicators. It is most important that the glass plates to which the thin layer is to be applied should be thoroughly clean and this can be achieved by washing the plates with concentrated sodium carbonate solution followed by thorough rinsing with distilled water.

The aqueous slurry of silica gel powder can be prepared by mixing about 15g of powder in $90cm^3$ distilled water and dispersing the powder for about one minute using a mechanical stirrer. The plates can then be activated by heating in an oven at $110^{o}C$ for one hour.

The sample solution to be applied should contain between 0.1 and 10mg of the sample per cm^3. About 1µl of solution is applied with a micro-syringe or micro-pipette near one end of the chromatoplate (about 1.5 to 2.0cm from the edge of the plate) and the latter air dried.

The chromatogram is usually developed by the ascending technique in

which the plate is immersed in the developing solvent (redistilled or chromatographic grade solvent should be used) to a depth of 0.5cm. The tank or chamber used is preferably lined with sheets of filter paper which dip into the solvent in the base of the chamber; this ensures that the chamber is saturated with solvent vapour. Development is allowed to proceed until the solvent front has travelled the required distance (usually 10 to 15cm), the plate is then removed from the chamber and the solvent front immediately marked with a pencil line.

The position of the separated solutes can be located by various methods. Coloured substances can be seen directly when viewed against the stationary phase while colourless species may usually be detected by spraying the plates with an appropriate reagent which produces coloured areas in the regions which they occupy. Some compounds fluoresce in ultraviolet light and may be located in this way.

The resulting chromatogram is described and the zones are characterised by R_f values. The R_f value is defined as the ratio of the distance (cm) from starting line to centre of zone : to the distance (cm) from starting line to solvent front. The R_f value measures the velocity of movement of the zone relative to the developer front. Those compounds of the mixture to be separated which are most readily soluble in the mobile phase will have R_f values near, or equal to, unity. Those components which have a lower solubility in the mobile phase will have R_f values near to zero. The R_f value is characteristic of a particular species in any given type of separation, and is sometimes used for the qualitative identification of an unknown species.

Extraction of Blankophor using TLC.

Much time has been spent during this investigation on methods to extract Blankophor and/or its metabolites from both distilled water and from filtered activated sludge water, with limited success.

It is not intended to develop an exact TLC separation procedure but merely to examine briefly TLC to see how readily separation might occur.

In all cases the TLC plates were prepared as follows: -

An aqueous slurry of silica gel powder was prepared by mixing silica gel powder (20g) with distilled water ($90cm^3$) and stirring. (5min.) The slurry was applied to clean glass plates (6 by 6in) (washed in concentrated sodium carbonate solution then distilled water). The plates were allowed to air dry (1hr.), placed in an oven ($110^{o}C$, 1hr.) then stored in a drying chamber (silica gel drying agent) and used as required.

The plates were spotted (micro-pipette, 1µl, 100ppm solution) with blankophor made up in filtered activated sludge water and allowed to air dry. All the plates were developed using the saturated ascending technique and the spots detected using an ultraviolet lamp.

Various methanol/water mixtures were examined as developing solvents: -

(a) 100% methanol,

(b) 75% methanol/25% water,

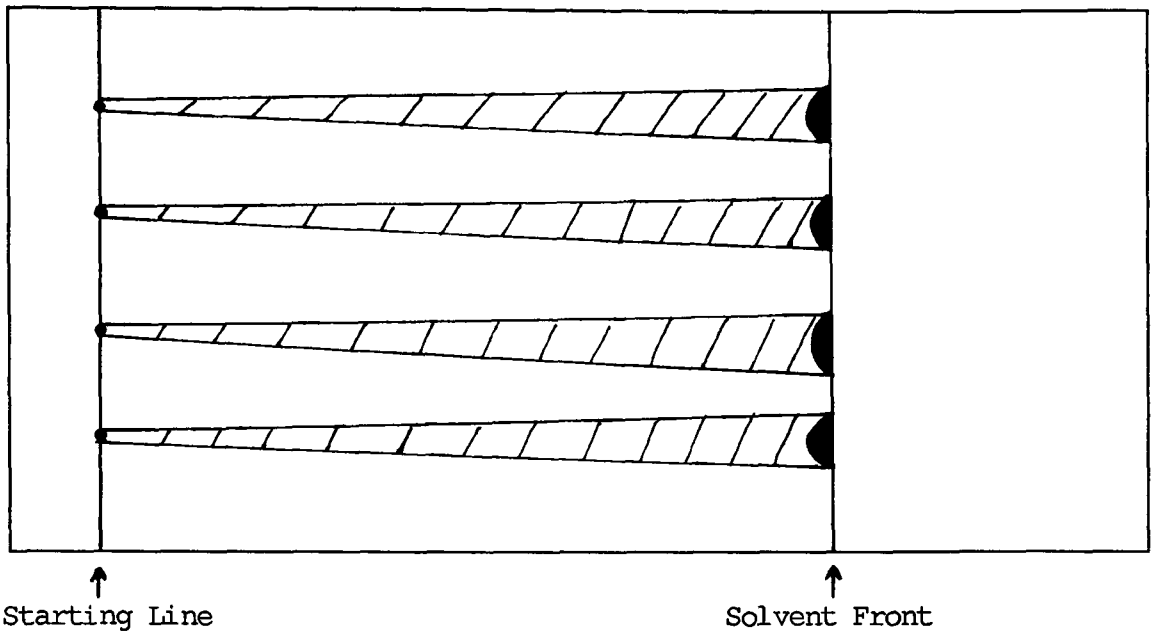
(c) 50% methanol/50% water,

(d) 25% methanol/75% water,

(e) 100% water.

The dried developed plates for all the methanol/water mixtures examined were identical. (Fig. 7.1)

Fig. 7.1 TLC PLATE, METHANOL/WATER AS DEVELOPING SOLVENT



The Blankophor solution travels with the solvent front, spreading out as it travels, leaving traces at the starting line and throughout the total length of the plate travelled, giving no clear separation.

The methanol/water may be too polar to separate the polar Blankophor from the interfering species in the filtered activated sludge water. The dielectric constant of methanol is 32.6 so other solvents with lower dielectric constants were sought.

Acetone and isopropyl alcohol (dielectric constants 17.0 and 18.3 respectively) were examined.

100% acetone (Fig. 7.2), 50% acetone/50% water (Fig. 7.3), 80% acetone/20% water (Fig. 7.4), and 90% acetone/10% water (Fig. 7.5) were examined as developing solvents.

Examination of Fig. 7.2 shows that using 100% methanol the solute spots do not move at the same velocity as the solvent front. There is some spreading of the solute spots and traces are left throughout the solute path giving no separation .

Fig. 7.2 TLC 100% ACETONE USED AS DEVELOPING SOLVENT

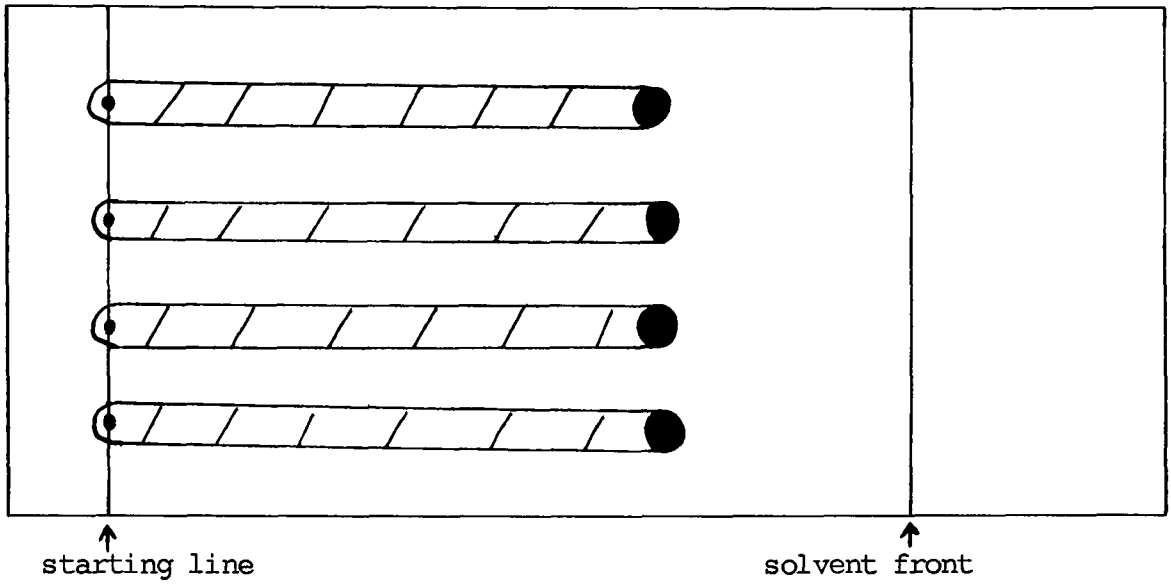
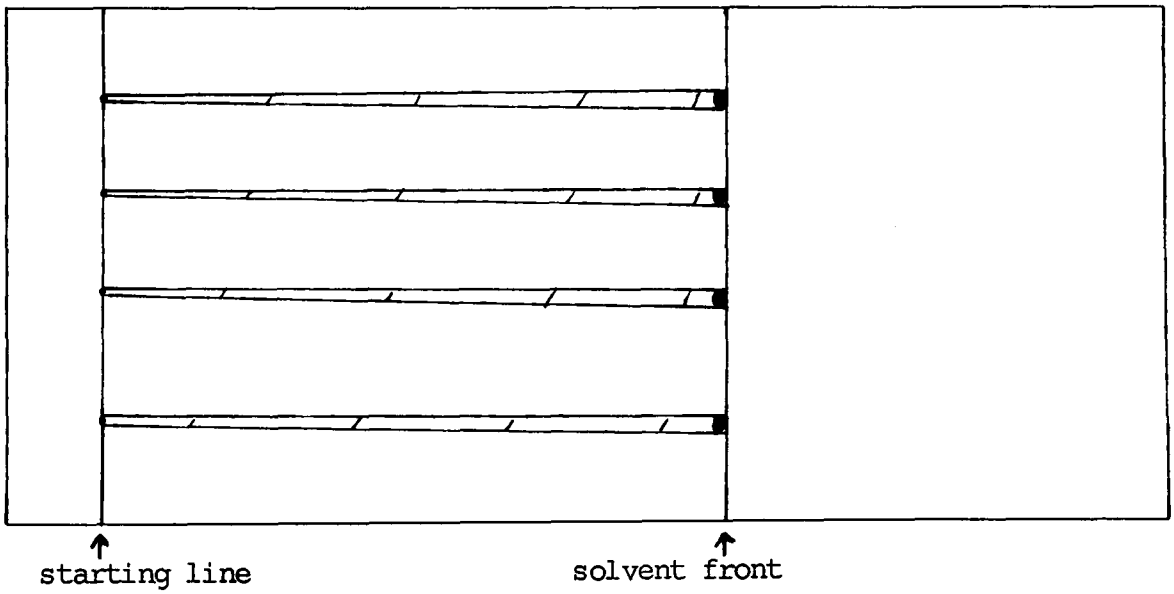
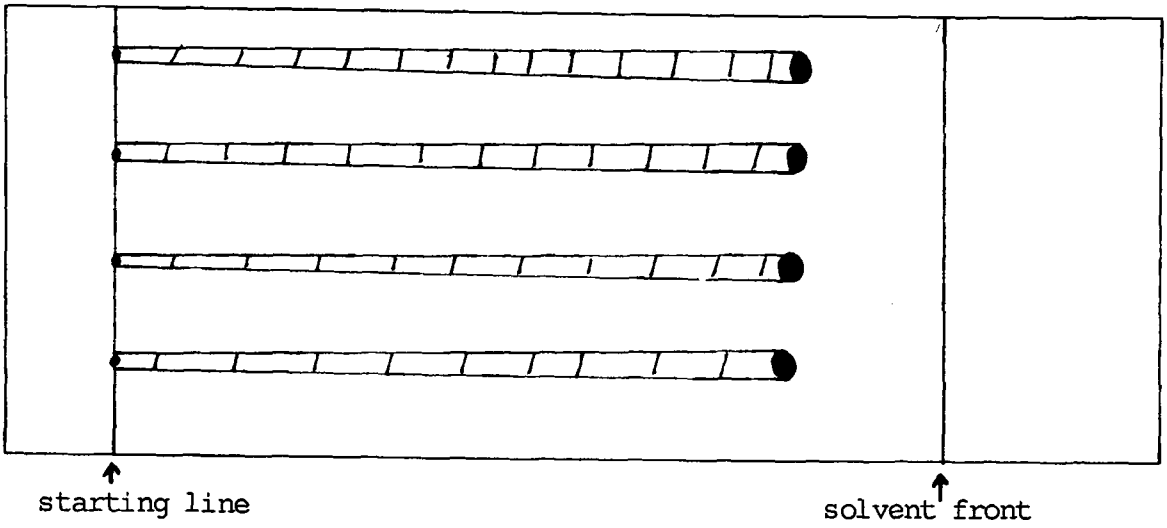


Fig. 7.3 TLC 50% ACETONE/ 50% WATER USED AS DEVELOPING SOLVENT



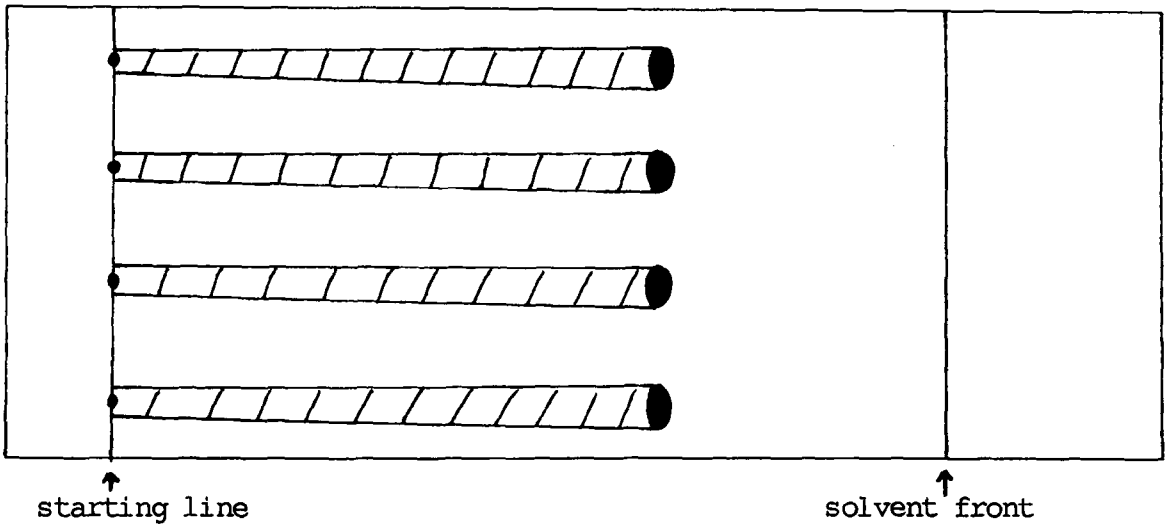
With 50% acetone/50% water as developing solvent the solute spots move at the same velocity as the solvent and there is much less solute left along the solute path, but again there is no indication of any separation taking place.

Fig. 7.4 TLC 80% ACETONE/20% WATER AS DEVELOPING SOLVENT



With 80% acetone/20% water as developing solvent the solute spots move at a slightly slower velocity than the solvent front, leaving traces of solute along the solute path, and there is no separation.

Fig. 7.5 TLC 90% ACETONE/10% WATER AS DEVELOPING SOLVENT



With 90% acetone/10% water as developing solvent the solute spots move at a slower velocity than the solvent front, leaving traces of solute along the solute path, and there is no separation.

Isopropyl alcohol was examined as a developing solvent but the results

obtained were virtually identical to those obtained for acetone. Because of the time restriction the TLC experiments were terminated at this stage.

The preceding investigations have examined the possibility of metabolites forming from biodegradation of the Blankophor. The Blankophor may undergo photo-degradation. It was thus decided to carry out photolysis experiments to see what, if any, metabolites were formed.

PHOTOLYSIS INVESTIGATIONS WITH BLANKOPHOR

PHOTOLYSIS INVESTIGATIONS WITH BLANKOPHOR.

Introduction:

The energy of photons in the visible and ultraviolet regions of the electromagnetic spectrum is great enough to produce electronic excitation. The reactions that occur as a result of absorption of photons are called photochemical reactions.

An excited molecule can undergo one of several processes. It can re-radiate the absorbed photon before it losses any energy in collisions with other molecules. Alternatively, an excited molecule can collide with other molecules and reach thermal equilibrium before emission. This process is called fluorescence.

If the excitation energy is high enough the excited molecule may react chemically or it may dissociate into excited fragments that themselves react chemically.

As previously discussed FWA's are substances which strongly absorb radiant energy in the near, ultraviolet region of the spectrum and re-emit it almost quantitatively in the form of longer wavelength visible blue light. It is therefor not unreasonable to expect some of the excited molecules to react chemically to yield photochemical metabolites.

Before undertaking a series of photolysis experiments a trial experiment was set up to determine sampling times.

Experiment 1.

Blankophor solution (10ppm in distilled water) in a quartz cell was placed under an ultraviolet lamp (Hanovia 1 litre PCR, 2 watt, 253.7 to 184.9nm). Ultraviolet absorbance readings were taken periodically (at T = 0, 15, 30, 60, 90, and 150 minutes) at various wavelengths (210 to 310nm) and recorded in TABLE 8.1. A distilled water blank was run

alongside the test solution.

Ultraviolet absorbance readings were measured (at T = 0) of a set of standard Blankophor solutions in distilled water (0 to 20ppm)(A1 to A9). The above procedure was repeated three times with absorbance readings being measured at 1, 2, 3T = 0, 30, 60 120, 180 and 240 min. (TABLE 8.1)(standard solutions B1 to B9, C1 to C9, and D1 to D9)

A SIMCA PLS model was generated using all four sets of standard Blankophor solutions and the Blankophor concentrations predicted for all the solutions (TABLE 8.2).

TABLE 8.1
Ultraviolet Absorbance of Blankophor Solutions.

Wavelength/nm (Absorbance Å x 10^{-3})											CONC.	OBJECT
210	220	230	240	250	260	270	280	290	300	310	PPM	NAME
26	59	85	92	89	94	97	97	97	97	98	0.0	A1
75	118	151	145	121	112	117	118	118	120	128	2.5	A2
147	185	217	197	153	131	136	139	137	141	157	5.0	A3
221	253	284	250	185	151	156	161	158	165	187	7.5	A4
282	331	361	290	216	198	230	236	204	191	208	10.0	A5
367	385	414	353	246	182	187	195	193	205	244	12.5	A6
436	458	486	403	278	212	227	236	224	229	271	15.0	A7
506	523	550	453	309	229	244	255	242	250	300	17.5	A8
576	590	617	504	340	251	270	283	265	273	328	20.0	A9
0	43	72	82	84	84	86	86	87	88	90	0.0	1B0
287	309	335	287	207	157	164	171	168	176	206	10.0	1T0
1	46	77	86	86	88	90	91	92	93	94	0.0	1B15
270	318	345	279	208	186	212	217	191	184	203	10.0	1T15
14	53	81	88	86	90	92	92	93	94	95	0.0	1B30
278	328	351	273	210	206	245	248	208	190	199	10.0	1T30
22	58	85	90	87	92	94	94	95	96	96	0.0	1B60
273	327	345	271	210	206	238	240	205	189	197	10.0	1T60
59	86	109	112	105	109	111	109	109	109	108	0.0	1B90
275	321	333	270	210	198	214	210	189	182	192	10.0	1T90
40	70	94	96	90	97	99	98	99	100	100	0.0	1B150
274	317	321	262	207	197	207	198	179	171	175	10.0	1T150
18	26	31	36	37	38	39	38	38	38	37	0.0	B1
87	91	96	86	67	55	58	58	57	58	65	2.5	B2
162	160	164	140	100	74	77	80	78	81	96	5.0	B3
230	223	227	190	128	89	92	96	94	101	124	7.5	B4
303	294	295	241	161	113	120	126	119	125	153	10.0	B5
375	356	358	294	190	124	128	135	132	143	181	12.5	B6
447	424	425	346	222	143	148	156	152	165	210	15.0	B7
519	492	492	398	254	163	169	180	174	187	239	17.5	B8

TABLE 8.1 CONT.

Wavelength/nm (Absorbance $\bar{A}$ x 10^{-3})											CONC.	OBJECT
210	220	230	240	250	260	270	280	290	300	310	PPM	NAME
589	557	557	448	284	180	187	198	191	207	267	20.0	B9
0	9	16	23	27	28	32	37	30	30	30	0.0	2B0
288	277	278	224	145	100	107	113	107	115	144	10.0	2T0
11	17	21	25	28	30	31	31	31	31	31	0.0	2B60
277	295	284	209	154	144	168	165	135	121	127	10.0	2T60
22	24	25	29	31	33	34	33	33	33	32	0.0	2B120
279	290	270	203	154	141	151	142	120	109	109	10.0	2T120
52	44	39	39	39	39	40	39	38	37	36	0.0	2B180
273	280	254	194	146	129	130	122	102	093	093	10.0	2T120
33	28	23	25	25	27	28	28	28	28	28	0.0	2B240
265	268	236	180	133	113	108	96	83	77	75	10.0	2T240
20	33	41	45	48	48	49	49	49	49	49	0.0	C1
93	100	106	97	77	65	67	68	68	70	78	2.5	C2
168	169	174	150	101	85	88	90	89	94	109	5.0	C3
237	233	237	200	138	101	104	108	107	114	138	7.5	C4
308	307	309	247	170	133	148	153	138	139	164	10.0	C5
382	367	370	304	200	136	140	148	145	157	196	12.5	C6
454	436	435	356	231	154	158	167	165	179	227	15.0	C7
526	501	501	407	261	172	176	187	183	200	255	17.5	C8
595	565	564	456	289	187	192	205	201	220	284	20.0	C9
0	16	26	33	38	39	42	41	43	43	43	0.0	3B0
286	281	286	234	155	110	116	124	119	128	159	10.0	3T0
15	26	30	36	39	40	42	42	43	43	43	0.0	3B60
275	298	286	217	167	154	167	162	140	130	136	10.0	3T60
23	29	31	35	38	39	41	41	42	42	42	0.0	3B120
271	288	265	208	160	139	136	124	111	105	105	10.0	3T120
24	29	30	33	36	37	38	38	39	40	40	0.0	3B180
269	281	248	193	147	123	112	98	87	83	80	10.0	3T180
50	49	43	48	49	48	49	48	48	49	48	0.0	3B240
292	293	251	193	145	119	103	88	80	75	72	10.0	3T240
1	20	28	35	38	40	41	41	41	42	41	0.0	D1
76	87	94	86	69	58	61	62	61	63	71	2.5	D2
149	155	161	138	100	77	79	83	81	85	101	5.0	D3
219	220	225	188	129	93	97	101	99	106	129	7.5	D4
288	294	297	234	160	128	146	151	133	131	154	10.0	D5
363	353	357	292	190	128	134	141	138	149	188	12.5	D6
435	420	423	344	221	146	152	161	157	170	218	15.0	D7
511	489	490	397	254	165	172	182	177	193	247	17.5	D8
576	551	549	449	275	179	183	201	198	213	276	20.0	D9
0	17	27	35	40	41	42	43	43	44	43	0.0	4B0
267	267	274	224	147	103	110	116	113	122	154	10.0	4T0
-11	5	14	23	27	30	31	32	33	34	34	0.0	4B60
247	274	262	203	155	142	153	145	124	115	118	10.0	4T60
-6	8	14	21	26	29	30	31	32	33	34	0.0	4B120
242	256	241	191	163	129	116	104	93	87	88	10.0	4T120
2	11	15	22	26	28	30	31	32	33	33	0.0	4B180
236	251	222	172	127	103	91	77	68	64	62	10.0	4T180
5	14	15	21	26	28	29	30	31	32	32	0.0	4B240
236	245	206	153	109	85	70	57	51	49	47	10.0	4T240

The PLS predicted concentrations for the sets of standard Blankphor solutions were very close to the standard values (-0.01 to +0.05ppm) and only the predicted values for the test solutions were recorded in TABLE 8.2.

TABLE 8.2
PLS Predicted Blankophor Concentrations:

OBJECT NAME	PLS CONC/PPM	OBJECT NAME	PLS CONC/PPM	OBJECT NAME	PLS CONC/PPM	OBJECT NAME	PLS CONC/PPM
T0	9.99	2T0	10.30	3T0	10.04	4T0	9.65
T15	9.75	2T60	9.97	3T60	8.83	4T60	8.55
T30	9.52	2T120	9.38	3T120	8.75	4T120	7.17
T60	8.95	2T180	8.52	3T180	9.14	4T180	7.74
T90	7.52	2T240	8.68	3T240	9.67	4T240	7.66
T150	6.82						

Examination of TABLE 8.2 indicated that a test period of four hours, with measurements taken at hourly intervals is a suitable procedure for photolysis.

Experiment 2.

A series of four Blankophor solutions (10ppm in distilled) were treated as per experiment 1, with absorbance measurements being recorded at T= 0, 60, 120, 180, and 240 minutes. A distilled water blank was carried out alongside each solution. In each case a set of standard Blankophor solutions (0 to 20ppm) were made up and absorbance readings taken at T = 0. (TABLE 8.3)

A SIMCA PLS model was generated using all four sets of standard Blankophor solutions and the Blankophor concentration predicted for all the solutions. The PLS predicted concentrations for the sets of standard Blankophor solutions were very close to the standard values (-0.04 to +0.05ppm with approximately 90% being exact) and only the predicted values for the test solutions were recorded (TABLE 8.4).

TABLE 8.3
Ultraviolet Absorbance of Blankophor Solutions.

Wavelength/nm (Absorbance $\tilde{A}$ x 10^{-3})											CONC.	OBJECT
210	220	230	240	250	260	270	280	290	300	310	PPM	NAME
0	0	0	0	0	0	0	0	0	0	0	0.0	A1
69	65	65	55	30	17	19	20	19	20	28	2.5	A2
144	134	133	104	63	36	38	42	40	43	59	5.0	A3
212	197	196	164	91	51	53	52	56	63	87	7.5	A4
285	268	264	205	124	75	82	88	82	87	116	10.0	A5
357	330	327	258	153	86	89	98	95	106	144	12.5	A6
429	398	394	310	185	105	109	118	114	127	173	15.0	A7
501	466	461	362	217	125	130	142	136	149	202	17.5	A8
571	531	526	412	247	142	148	160	153	179	230	20.0	A9
0	0	0	0	0	0	0	0	0	0	0	0.0	B0
261	253	253	196	112	62	68	76	71	81	111	10.0	T0
11	8	5	2	1	2	3	4	3	3	3	0.0	1B60
250	271	259	181	121	106	129	128	99	87	94	10.0	1T60
22	15	9	6	4	5	6	6	5	3	4	0.0	1B120
252	266	245	175	121	103	112	105	84	75	76	10.0	1T120
52	35	23	16	12	11	12	12	10	7	8	0.0	1B180
246	256	229	166	113	91	91	85	66	59	60	10.0	1T180
33	19	7	2	0	0	0	1	0	0	0	0.0	1B240
238	224	211	152	100	75	69	59	47	43	42	10.0	1T240
0	0	0	0	0	0	0	0	0	0	0	0.0	B1
73	67	65	52	29	17	18	19	19	21	29	2.5	B2
148	136	133	105	62	37	39	41	40	45	60	5.0	B3
217	200	196	155	90	53	55	59	58	65	89	7.5	B4
288	274	268	202	122	85	99	104	89	90	115	10.0	B5
362	334	329	259	152	88	91	99	96	108	147	12.5	B6
434	402	395	311	183	106	110	158	116	130	178	15.0	B7
506	468	460	362	113	154	127	138	134	151	206	17.5	B8
575	532	523	411	244	139	143	156	152	171	235	20.0	B9
0	0	0	0	0	0	0	0	0	0	0	0.0	2B0
284	265	261	202	118	72	76	84	77	86	117	10.0	2T0
15	10	4	3	2	1	0	1	0	0	0	0.0	2B60
273	282	261	185	130	116	127	122	99	88	94	10.0	2T60
23	13	5	2	0	0	-1	0	-1	-1	-1	0.0	2B120
269	272	240	176	142	100	94	83	68	62	62	10.0	2T120
24	13	4	0	-2	-2	-4	-3	-4	-3	-3	0.0	2B180
267	265	223	161	110	84	72	58	45	41	38	10.0	2T180
50	33	17	15	11	9	7	7	5	6	5	0.0	2B240
290	277	226	161	108	81	63	48	38	33	29	10.0	2T240
0	0	0	0	0	0	0	0	0	0	0	0.0	C1
75	67	66	51	31	18	20	21	20	21	30	2.5	C2
148	135	133	103	62	37	38	42	40	43	60	5.0	C3
218	200	197	153	91	53	56	60	58	64	88	7.5	C4
287	274	269	199	122	88	105	110	92	89	113	10.0	C5
362	333	329	257	152	88	93	100	97	107	147	12.5	C6
434	400	395	309	183	106	111	120	116	128	177	15.0	C7
510	469	462	362	216	125	131	141	136	151	206	17.5	C8
575	531	521	414	237	139	142	160	157	171	235	20.0	C9
0	0	0	0	0	0	0	0	0	0	0	0.0	3B0
284	265	260	201	117	71	76	81	77	85	117	10.0	3T0

TABLE 8.3 CONT.

Wavelength/nm (Absorbance Å x 10^{-3})											CONC.	OBJECT
210	220	230	240	250	260	270	280	290	300	310	PPM	NAME
-12	-12	-13	-12	-13	-11	-11	-11	-10	-10	-9	0.0	3B60
264	272	248	180	125	110	119	110	88	78	81	10.0	3T60
-6	-9	-13	-14	-14	-12	-12	-12	-11	-11	-9	0.0	3B120
259	254	227	168	133	97	82	69	57	50	51	10.0	3T120
2	6	-12	-13	-14	-13	-12	-12	-11	-11	-10	0.0	3B180
253	249	208	149	97	71	57	42	32	27	25	10.0	3T180
5	-3	-12	-14	-14	-13	-13	-13	-12	-12	-11	0.0	3B240
253	243	192	130	79	53	36	22	15	12	10	10.0	3T240
0	0	0	0	0	0	0	0	0	0	0	0.0	D1
72	67	67	52	31	18	20	22	20	22	30	2.5	D2
139	133	132	101	61	39	46	49	42	43	57	5.0	D3
212	198	196	153	91	53	57	61	57	63	86	7.5	D4
275	281	276	193	124	108	142	147	108	93	107	10.0	D5
357	332	329	257	153	89	94	102	96	106	145	12.5	D6
430	400	395	309	184	106	112	121	116	128	174	15.0	D7
503	469	463	362	216	127	135	146	137	150	203	17.5	D8
573	536	529	412	247	148	158	170	159	173	233	20.0	D9
0	0	0	0	0	0	0	0	0	0	0	0.0	4B0
284	266	261	202	118	72	77	82	78	87	118	10.0	4T0
12	7	2	1	1	-1	-1	0	0	-1	0	0.0	4B60
272	275	244	179	127	106	103	90	74	67	69	10.0	4T60
17	10	3	1	0	-1	-1	-1	-1	-2	-1	0.0	4B120
271	271	235	171	120	97	89	75	61	53	53	10.0	4T120
2	12	3	0	-1	-3	-2	-1	-2	-2	-1	0.0	4B180
283	275	229	165	112	86	71	56	46	41	38	10.0	4T180
30	20	6	2	0	-1	-1	-1	-2	-2	-1	0.0	4B240
268	256	205	140	89	62	45	31	23	19	16	10.0	4T240

TABLE 8.4
PLS Predicted Blankophor Concentrations.

OBJECT NAME	PLS CONC/PPM	OBJECT NAME	PLS CONC/PPM	OBJECT NAME	PLS CONC/PPM	OBJECT NAME	PLS CONC/PPM
1T0	9.41	2T0	9.97	3T0	9.98	4T0	10.01
1T60	9.01	2T60	8.97	3T60	8.98	4T60	9.25
1T120	8.50	2T120	7.74	3T120	7.81	4T120	8.24
1T180	7.35	2T180	7.28	3T120	8.16	4T120	8.20
1T240	7.27	2T240	6.96	3T240	8.01	4T240	8.01

Examination of TABLE 8.4 shows that the Blankophor concentration fell, in all four test solutions, during the test period of four hours. With the exception of test 3, the concentration fell gradually by 2 to 3ppm. Test 3 fell gradually at first then levelled out.

A further experiment was carried out (as in experiment 2) to see if the above results were reproducible. The PLS predicted Blankophor concentrations for the test solutions are shown in TABLE 8.5.

TABLE 8.5
PLS Predicted Blankophor Concentrations.

OBJECT NAME	PLS CONC/PPM	OBJECT NAME	PLS CONC/PPM	OBJECT NAME	PLS CONC/PPM	OBJECT NAME	PLS CONC/PPM
1T0	9.87	2T0	10.01	3T0	9.89	4T0	9.92
1T60	8.74	2T60	9.05	3T60	8.74	4T60	8.89
1T120	8.12	2T120	8.34	3T120	8.21	4T120	8.09
1T180	7.74	2T180	7.85	3T180	7.93	4T180	7.82
1T240	7.51	2T240	7.69	3T240	7.74	4T240	7.53

As with the biodegradation experiments, the "fit" of the SIMCA PLS model predictions for the test solutions was good with that of the standards being excellent (this was as expected as distilled water does not contain any interfering species unlike filtered activated sludge solution).

However, it could be seen, by visual inspection, that the ultraviolet spectrum of the test solutions changed during the test period.

Graphs of absorbance versus wavelength were constructed (FIG. 8.1) for the test solutions, and for the 10ppm standard solutions (FIG. 8.2), in experiment 2.

Examination of FIG. 8.2 shows that there is very little change in the spectrum of the 10ppm standard Blankophor solution. FIG. 8.1 shows a definite change in the spectrum of the test solution during the test period in the regions 210 to 230nm, and 260 to 310nm.

The above change in the test spectrum and the fall in Blankophor concentration indicates that some of the Blankophor has undergone photolytic conversion to metabolites and/or isomers.

It should be noted that the test solutions were irradiated with high energy ultraviolet radiation and any photolysis which occurred is unlikely in the United Kingdom as the weather here is unlikely to produce radiation of such energy. However, there are many parts of the world were such high energy radiation does fall and photolysis of FWA'S can occur.

Fig. 8.1 Ultraviolet Spectra of Test (1) Solution from Experiment 2.

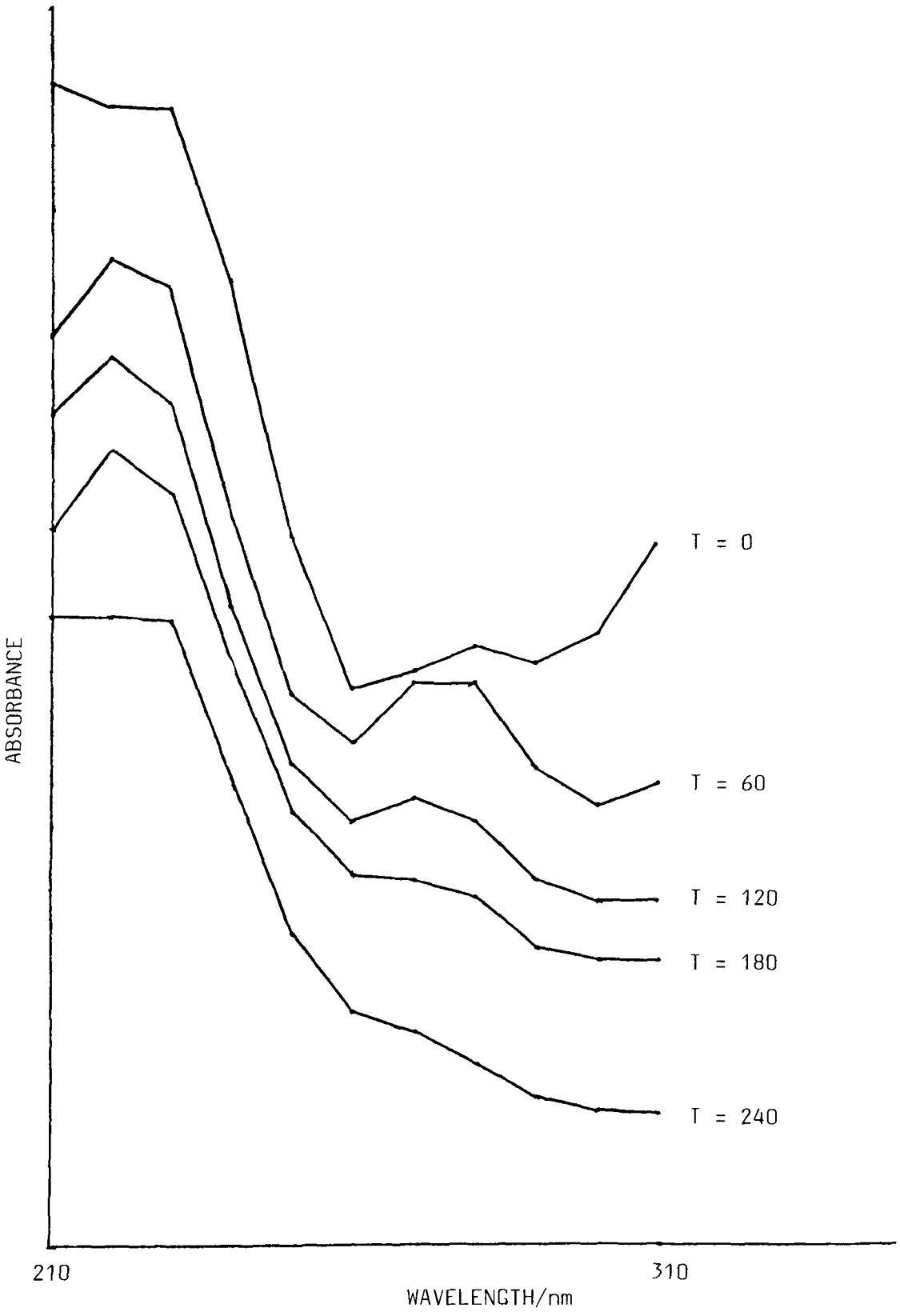


Fig. 8.2 Ultraviolet Spectra of 10ppm Standard Blankophor Solutions from Experiment 2.

DISCUSSION AND CONCLUSIONS.

DISCUSSION AND CONCLUSIONS:

During the past decade public awareness and concern has increased dramatically over the impact man has on his environment, as shown by increased support for environmental pressure groups such as Friends of the Earth and the Green Party. Articles expressing concern about environmental issues are appearing regularly in the Local and National Press, and television programmes dealing with these issues are fairly commonplace.

As a result of this increased awareness environmental issues have been placed high on the "Political Agenda", and more and more emphasis has been placed on the need to examine benefit against any hazard involved in the use of new chemicals and technology.

Fluorescent whitening agents (FWA's) have been used in domestic and industrial premises for many years. FWA's are widely used in the textile, plastics, photographic, synthetic fibres and soaps and detergents industries.

As the name implies FWA's are used to produce a brilliant white finish to textiles and paper. Industry has changed to using FWA's because it has concluded that the overall cost of using these compounds is less than that of other bleaching materials and the white produced is much brighter and "whiter".

The main benefit to the consumer from the use of FWA's is an aesthetically pleasing bright white colour.

An attempt was made, in 1975, to assure the public about the safety of FWA's by the publication of; Fluorescent Whitening Agents - Environmental Quality and Safety Supplement Vol. 4, which, in the words of the editors, set out to present in a concise form all the then known facts about FWA's.

In this publication the editors stated in their Foreword that FWA's had been fully tested long before questions about the environment were raised, and that, thorough toxicity testing had rapidly eliminated the few problem products.

The editors also stressed the findings of a symposium at the Swedish Centre for Environmental Sciences, attended by specialists from Government Agencies, Universities and Industry. The symposium came to the conclusion that FWA's posed no hazard to man or the environment.

However, the editors also stressed that, by their strong fluorescence, FWA's could easily be detected even at extremely low levels.

It has been found[14] that, with the stilbene-s-triazine type FWA's, fluorescence is largely lost by conversion from the trans to the cis isomer, and hence many of the findings regarding removal and dispersion of these FWA's, as measured by fluorescence spectroscopy, could prove to be totally meaningless.

Also, little was known about the biodegradability of FWA's or their primary degradation products. Because of the presence of the many amino groups in stilbene-s-triazine type FWA's there was concern that biodegradation of these FWA's could lead to metabolites exhibiting oncological effects.

It was due to these concerns, and the lack of information on the biodegradability of FWA's, coupled with the loss of fluorescence by trans to cis isomerisation and what this implies, that this investigation was instigated.

Many toxicological investigations have been carried out with FWA's. It has been estimated by Buxtorf[42] that, excluding occupational hazards, in the extreme case a persons maximum daily intake of FWA's (orally and by

skin contact) was 4.4µg/kg.

Thomann and Kruger[43] carried out acute oral, dermal and inhalation studies with thirty six FWA's, including the stilbene-s-triazine types. They concluded that the test compounds were non toxic, that they caused no primary skin irritation, and that they caused no local changes in the mucosae of the nose and eye.

The above results and those summarised in Appendix 1., and detailed in: Fluorescent Whitening Agents pages 190 to 277, suggested that there is little or no toxicological danger involved in the use of FWA's.

Keplinger et al[44] and others [45, 46,] examined some twenty FWA's to assess their mutagenic properties. They all concluded that the FWA's tested showed no evidence of mutagenic activity.

Bioaccumulation studies were undertaken by Hamburger [27] and others [26, 47], using ^{14}C labelled FWA's, on fish, rats, and bean plants.

The studies on fish and rats indicated that there was no evidence of bioaccumulation of any of the five FWA's tested. In the bean plant studies there was evidence that the three ^{14}C labelled FWA's were rapidly accumulated by the root material, but there was only a very limited translocation to the leaves and bean pods. It was concluded that it was very unlikely that, under practical conditions, residues of the FWA's appeared in any substantial amounts in the edible parts of crops. Sludge deposits from sewage treatment plants are often spread on agricultural land. The results obtained from the bean plant studies indicate that any FWA's adsorbed onto this solid material would not find its way into the food chain via the edible parts of food crops.

Akamatsu [48] and others [49, 50, 51] carried out photolysis experiments on some twelve FWA's in aqueous solutions. They all concluded that the loss of fluorescence was due to the trans to cis photoisomerisation in the

stilbene groups of the molecule and not to photodegradation.

If degradation of stilbene-s-triazine type FWA's occurs, it is likely to occur at the double bond of the stilbene group of the molecule. (this is supported by evidence from ^{14}C studies carried out for The Thames Water Authority)

One possible metabolite resulting from the biodegradation of stilbene-s-triazine FWA's at the stilbene double bond is the corresponding carboxylic acid. In the initial part of this investigation a model carboxylic acid metabolite [2,4-bis-diethyleneamino-6-(5-amino-2-carboxy benzene sulphonic acid)-s-triazine] was synthesised.

During the synthesis of this model metabolite six compounds were synthesised. They included:

(1) 2,4-bis(diethylamino)-6-chloro-s-triazine,

(2) 2-sulphonic acid-4-nitro toluene,

(3) 4-nitro-2-sulpho benzoic acid,

(4) 5-amino-2-carboxy benzene sulphonic acid,

(5) 2,4-bis(diethylamino)-6-(5-amino-2-carboxy-benzene sulphonic acid)-s-triazine,

(6) 2,4-bis(diethylamino)-6-(4-amino benzoic acid)-s-triazine.

Infrared and N.M.R. spectra were obtained for all six compounds (Appendix 3 and Appendix 4) along with their melting/boiling points (Chapter 1). Mass spectra were obtained for compounds 1, 4, 5 and 6 (Appendix 5). Limited mutagenicity tests were also carried out on compounds 1, 4, 5, and 6 (Appendix 2).

Biodegradation studies (Chapter 2), using HPLC, on the model carboxylic acid metabolite, produced no evidence that this primary degradation product of a stilbene-s-triazine type FWA underwent any further

degradation. There was no indication that the compound was adsorbed onto the solid material of the activated sludge solution and no evidence that the sulphonate group was removed (the presence of a sulphonate group tends to detoxify many compounds). Indeed it would appear that this compound was much more stable than was at first suspected.

A purified commercial stilbene-s-triazine type FWA; Blankophor REU-P; was obtained from the Thames Water Authority. An analytical method, involving ultraviolet absorbance measurements at various wavelengths followed by SIMCA computer modelling, was developed to determine the concentration of the Blankophor REU-P in filtered activated sludge solution, prior to undertaking biodegradation studies.

The predictive ability of this analytical method in determining the concentration of Blankophor REU-P in filtered activated sludge solution was excellent. In the majority of cases (approximately 90%) the predicted concentrations were within ± 1.0% 0f the calculated values. In the remaining cases some 95% of the predicted concentrations were within -2.0% to +4.0% of the calculated concentrations, with the remaining 5% being within ± 9.0% of the calculated concentrations.

Biodegradation studies were carried out with the Blankophor REU-P stilbene-s-triazine FWA, in activated sludge solution, using this analytical method to monitor Blankophor REU-P concentration.

During the test period (six days) the Blankophor REU-P concentration fell, in all the test solutions, from 10ppm to approximately 5.5ppm indicating that Blankophor REU-P was being removed or changed in some way.

The ultraviolet spectra of the filtered test solution changed shape during the test period, especially in the region 210nm to 260nm, indicating that the fall in Blankophor REU-P concentration was indeed

due, in part at least, to some change in its composition.

It was not possible at this stage of the investigation to establish exactly what was occurring to the Blankophor REU-P FWA, but it was possible to make the following deductions:

1. Blankophor REU-P was not being adsorbed onto the solid material of the activated sludge solution:- degradation studies in Chapter 2, and ^{14}C studies [29] carried out on behalf of the Thames Water Authority support this deduction,
2. The change in the ultraviolet spectra of the filtered Blankophor REU-P solutions was not due to metabolism of any of the compounds contained in the raw activated sludge solutions:- if this had been the case then the ultraviolet spectra of the blank solutions would also have changed but this did not occur,
3. The Blankophor REU-P had been metabolised and/or isomerised:- supported by the fall in Blankophor REU-P concentration coupled with the changes in the ultraviolet spectra, and points 1 and 2 above.

Attempts were made to develop a method to separate the Blankophor REU-P and the metabolites or isomers from the six day biodegradation solutions, with a view to separating them into their component compounds and identifying them. Methods were examined that might extract the Blankophor REU-P from the filtered activated sludge solution, making the assumption that any technique which extracted the Blankophor REU-P would also extract any metabolite or isomers.

As the Blankophor REU-P was in the sodium form, ion-pairing at low pH (2.0) was considered as a possible separation technique. Two ion-pairing reagents; tetraphenyl arsonium chloride (a well known ion-pairing reagent) and poly[oxyethylene(dimethylimino)-ethylene(diethylimino)-

ethylene] (WSCP) (a newer polymeric material supplied by Buckman Laboratories Inc.) were examined.

Continuous extraction, using ethyl acetate; isobutyl alcohol; acetylacetone; and tetrachloro methane as solvents; with both ion-pairing reagents produced no extraction of the Blankophor REU-P FWA.

Thin layer chromatography was examined briefly, using silica gel plates and various solvents and solvent mixtures, but no successful separation was achieved. In the absence of any suitable separation technique further biodegradation studies with the Blankophor REU-P were abandoned.

Since FWA's are substances which strongly absorb radiant energy in the ultraviolet region of the spectrum it might be expected that they would undergo photodegradation. Hence, photolysis experiments were undertaken with the Blankophor REU-P FWA in distilled water.

The concentration of the Blankophor REU-P fell, in all the test solutions, from 10ppm to an average of 7.5ppm over the test period. The ultraviolet spectra of the test solutions changed shape during the test period, indicating that the Blankophor REU-P had undergone photolytic conversion. It was not possible, however, to identify which products had been formed. As with the biodegradation experiments metabolites and/or isomers may have been produced.

One possible explanation for the results obtained in the biodegradation and photolysis experiments was that, in the biodegradation studies the stilbene bond was broken, giving rise to compounds of the type investigated in Chapter 2 which are stable and do not readily undergo further degradation, and that, in the photolysis experiments there was a trans to cis isomerisation about the stilbene bond. However, it must be stressed that this investigation has not supplied sufficient evidence to verify this and this hypothesis is based largely on the work carried out

by other researchers.

Much more research will have to be undertaken before complete illumination is obtained regarding the fate of FWA's in the environment. The compounds synthesised in Chapter 1 are all possible metabolites from the degradation of stilbene-s-triazine type FWA's. The limited mutagenicity testing (one in vitro point assay with two strains of Salmonella) on four of these compounds indicated no signs of any oncological effects. However, this is not sufficient to conclusively state that these compounds do not exhibit mutagenicity. The Department of Health Report 35 - Guidelines for the Testing of Chemicals for Mutagenicity HMSO 1989, requires the quoting of two in vitro and one in vivo test results.

Although it is clear that FWA's are fairly stable compounds which are slow to bio- and photo- degrade and that their toxicity to man, both directly and by bioaccumulation is minimal, little is still known about the toxicity of possible degradation products.

FUTURE WORK.

FUTURE WORK:

As discussed in the previous section little is still known about the toxicity of possible degradation products of FWA's and mankind is concerned about the possible effects of these chemicals on himself and on his environment.

How can this concern be alleviated ?

FWA's are used solely to satisfy aesthetic criteria and at first glance the simplest solution would appear to be a total ban on the use of FWA's until such times as the toxicity of all the possible degradation products could be determined. However, this research could prove to be very expensive and who would pay , the consumer, industry or the government.

Perhaps it would be best never to use these chemicals again, surely drab grey/yellowish textiles and paper would be a small price to pay for ease of mind regarding the possible damage these chemicals may have on man and his environment.

However, this again is not as simple as it would at first appear because man places a strong emphasis on the colour White.

Lusher [52] investigated the psychological aspects of white. In a representative study involving 1994 respondents 95% of them attributed whitening to a fluorescent whitened white, whereas 82% denied this attribute to non-fluorescent white. He suggested that white acts as a visual stimulus on the wakefulness centre of the formatio reticularis on the brain stem, and in the event of tension building up to crisis and conflict a subcortial unconscious decision is made in favour of flight, liberation and freedom.

He concluded that freedom is the basic idea in the emotional significance of white, and is expressed in terms of liberation from

constraint. The idea of purity implies being free from alien qualities, generally substances of inferior value (such as dirt). Freedom from sin and error implies innocence, chastity (as symbolised in the bridal veil) and sanctity. White signifies freedom from conflict as the colour of peace (in the white flag) and of truth (in the white book). White also signifies freedom to explore new possibilities, to make a new start.

As an emotional representation of the feeling of freedom, white responds to a psychological need in human beings. This explains its popularity and widespread use.

White is the only colour that can stand for freedom as an emotion and as a psychic need. It is thus hard to believe that modern man would accept the ban on FWA's and the resulting non-white paper and textiles.

Another possibility would be to limit the numbers of FWA's in current use by elimination of those FWA types which "appear to present the most hazard."

Although some 4,000 commercial brands of FWA's are known [53] a closer look will reduce this figure to about 250 important brands from about 50 chemical individuals.

These 50 chemical individuals can be traced back to 15 types of compounds which are partly water soluble and partly dispersible and which can be derived from the following 6 major structural types:-

(A) Stilbene derivatives,

(B) Coumarin and carbostyril compounds,

(C) 1,3-Diphenyl-2-pyrazolines,

(D) Naphthalimides

(E) Benzayolyl substitution products of ethylene, phenyl ethylene, stilbene and thiophene,

(F) Combined heteroaromatics - thiazoles, pyrazoles, oxdiazoles, triazines - with ethylene or aromatic systems.

Many of the compounds of the type B to F contain sub-structures involving pyrene and other similar structures [54] which, if they undergo degradation, are likely to be far less environmentally friendly than the stilbene-s-triazines.

If these types of FWA's were to be eliminated from current use and only the "safer looking" stilbene-s-triazines used then the "potential hazard" would be greatly reduced.

Probably the only way in which the concern about the possible hazards involved in the use of FWA's will be alleviated is if this concern is sufficient to force the consumer, industry and the Government into providing the funds necessary for further research into the toxicology of possible metabolites from Fwa's in current use.

APPENDIX 1.

TOXICOLOGICAL DETAILS OF FWA's.

Many toxicological investigations have been carried out with FWA's. It has been estimated by Buxtorf [42] that, excluding occupational hazards, in the extreme case a persons maximum daily intake of FWA's (orally and by skin contact) was 4.4μg/kg.

Thomann and Kruger [43] carried out acute oral, dermal and inhalation studies with thirty six FWA', including the stilbene-s-triazine types. They concluded that the test compounds were non-toxic, that they caused no primary skin irritation, and that they caused no local changes in the mucosae of the nose and eye.

Luckhaus[55] studied the effect of a stilbene type FWA on the skin of mice. He concluded that the FWA did not penetrate into the subepithelial layers (dermis and subcutaneous tissue) of the skin after cutaneous application, and that any risk associated with the use of FWA's could be ruled out, as there is no evidence of adsorption into the body after skin contact.

Keplinger et al [56]carried out two year feeding studies, on albino rats and beagle dogs, with four FWA's including two stilbene-s-triazine types, to determine the effects of long term, high-level exposure to these agents.

Weekly dietary levels up to 1000ppm caused no adverse toxic effects in albino rats and weekly dietary levels up to 2000ppm caused no adverse toxic effects in beagle dogs.

Lorke and Machemer[57] carried out embryo toxicity tests on rats and rabbits with two FWA's including one stilbene-s-triazine type. They found that the two FWA's were well tolerated by rats and dogs during pregnancy and that they had no harmful effects at doses up to 1000mg/kg/day. They also found that there was no indication of embryo toxicity in either species.

FWA's have been subjected to extensive toxicological tests on a scale usually reserved for substances like pesticides with which the risk of exposure and intake is infinitely greater, from the intended application and biological activity of which it must be assumed that they are apt to give rise to adverse effects.

The outcome of the tests was invariably the same: all the experimental investigations carried out confirmed that the safety margin for every conceivable application of FWA's is very wide and actually far greater than is demanded by international standards for medicaments, pesticides, food additives, etc. The likelihood of Fwa's constituting a toxicological danger to human health is therefore so minimal as to be completely negligible.

APPENDIX 2.

MUTAGENICITY TESTS.

The mutagenicity tests on the compounds synthesised in Chapter 1 were carried out, on behalf of The Thames Water Authority, by The Robens Institute of Industrial and Environmental Health and Safety, University of Surrey.

Mutagenicity Tests on the Compounds Synthesised in Chapter 1.

Introduction

Many organic chemicals are thought to cause cancer by altering the genetic apparatus - deoxyribonucleic acid (DNA) - of the cell. Chemicals may be either already chemically active or converted to active species in the body (metabolic activation). The interaction of a chemical with bacterial DNA may be a rapid and simple indication of Carcinogenic potential. The bacterial mutagenicity test, often referred to as the Ames test, is used to identify point mutation in several mutant strains of Salmonella typhimurium.[58, 59] These mutant bacteria require histidine to grow and a mutation is indicated by a reversion of the bacteria to grow on medium deficient in histidine.

The bacteria strains allow different types of mutation to be detected. The five salmonella typhimurium strains used are all derived from Salmonella typhimurium LT_2. These strains also carry other mutations making them more susceptible to the mutagenic effect of chemicals. A deletion through the uvrB region of the chromosome eliminates the excision repair system for DNA. This deletion includes the gal and bio region resulting in an absolute requirement for biotin and partial loss of the lipo-polysaccharide that coats the bacteria. A further mutation, rfa, results in further loss of the lipo-polysaccharide coat. The loss of this coat makes the bacteria more permeable to large lipophobic molecules.

The Salmonella typhurium strains used do not metabolise most chemicals to any significant extent and a metabolising system, typically a rat liver preparation, is therefore added to enable chemicals to be metabolically activated. In an attempt to optimise the formation of active metabolites it is common to use a microsomal preparation from

rats pretreated with Aroclor 1254. This fraction contains the mixed function oxidase enzymes which act as terminal oxidases for the oxidative reactions which frequently metabolically active chemicals.

Summary of the Report from the Robens Institute

Three aqueous (5%) samples (1). [2,4-bis-(diethylamino)-6-(5-amino-2-carboxy-benzenesulphonic acid)-s-triazine](pH 7.1), (2). [2,4-bis-(diethylamino)-6-(4-amino-benzoic acid)-s-triazine](pH 8.0), (3). [5-amino-2-carboxy-benzenesulphonic acid](pH 6.5) and one dimethylformamide (DMF)(5%) sample (4). [2,4-bis-(diethylamino)-6-chloro-s-triazine] were tested for mutagenic activity in Salmonella typhimurium TA98, TA100, TA1538, TA1535, and TA1537 without and with metabolic activation by liver S9 fraction from Aroclor-induced rats.

The sample size limited the highest dose tested for (1), (2), and (3), they were tested up to 5000µg plate in the following strains:-

(1) in TA98, TA1537, TA1535, and TA1538

(2) in TA98, TA1537, TA1535

(3) in TA1537, TA1538, TA1535

The highest dose of (4) was limited to 1000µg plate to prevent toxicity of the DMF towards the bacteria. Sample (1) was found to be contaminated and therefore was filter sterilised prior to use. Visible precipitate was observed at 5000µg/plate with (2), while under the light microscope precipitate was observed at 1000 and 300µg/plate.

No sample exhibited mutagenic activity in any strain tested, either with or without metabolic activation at the doses tested in this study.

A single experiment in A98 and Ta100 with a 20min preincubation step was carried out on (1), (2) and (3). None of these samples exhibited mutagenic activity in this experiment.

Lorke and Mackener [45] carried out mutagenicity studies on five FWA's using the dominant lethal test on male mice. They concluded that there was no evidence of mutagenicity due to these FWA's.

Muller et al [46] tested four FWA's for mutagenic activity after oral administration in the following three different mammalian test systems:

(1). Dominant lethal test in the male mouse,

(2). Cytogenetic studies on metaphase chromosomes from the bone marrow of the Chinese hamster,

(3). Nucleus anomaly test in bone marrow cells of the Chinese hamster.

The results obtained in all these tests gave no indication of mutagenic activity after the administration of the FWA's in dosages up to approximately 1/3 of the LD_{50}.

Kilbey and Zetterburg [60] carried out mutagenicity assays on six FWA's. With Salmonella typhimurium, using the method of Ames et al [61], the six FWA's failed to elicit a mutagenic response in the presence of rat liver postmitochondrial supernatant and cofactors. They also examined their activity as inducers of cytoplasmic petite mutants and mitotic gene conversion in diploid yeast (Saccharomyces cerevisiae) and reversion from auxotrophy to prootrophy in Neurospora crassa, Escherichia coli, and Salmonella Typhimurium. The results provided no indication that they produce mutagenic alterations in the gene material.

As with the toxicity tests the extensive work carried out on FWa's indicates that they show no signs of mutagenicity.

APPENDIX 3.

I. R. SPECTRA.

Introduction:

It is not intended to give a definitive explanation of I.R. spectroscopy: there are many textbooks available on the subject [62, 63] but merely to give some background information prior to interpreting some spectra.

When two atoms combine to form a stable covalent molecule (eg HCl gas) they may be said to do so because of some internal electronic rearrangement and we can simply look on the phenomenon as a balancing of forces. On the one hand there is a repulsion between the positively charged nuclei of both atoms, and between the negative electron "clouds"; on the other there is an attraction between the nucleus of one atom and the electrons of the other, and vice versa. The two atoms settle at a mean internuclear distance such that these forces are just balanced and the energy of the whole system is at a minimum. Attempt to squeeze the atoms more closely together and the repulsive force rises rapidly; attempt to pull them further apart and we are resisted by the attractive forces. In either case an attempt to distort the bond requires an input of energy.

The compression and extension of a bond may be likened to the oscillations of a spring. If the energy is increased the oscillation will become more vigorous but the vibrational frequency will not change. (an elastic bond, like a spring, has a certain vibrational frequency dependent upon the mass of the system and the force constant but independent of the amount of distortion)

Vibrational energies, like all other molecular energies, are quantised and the allowed vibrational energies for any particular system may be calculated from the Schrodinger equation.

Vibrational energy changes will only give rise to an observable spectrum

of the absorption bands. Thus these bands are often referred to as the "fingerprint" bands because a molecule or structural moity may often be recognised from the appearance of this part of the spectrum.

Group frequencies, on the other hand, are usually almost independent of the structure of the molecule as a whole, and, with a few exceptions, fall in the region well above and well below that of the skeletal modes. Vibrations of light atoms in terminal groups (e.g. $-CH_3$, -OH, -C=O, =C=C, etc.) are of high frequency, while those of heavy atoms (eg -C-Cl, -C-Br, metal-metal, etc.) are low in frequency. Their frequencies, and consequently their spectra, are highly characteristic of the group, and can be used for analysis.

In summary, then, experience coupled with comparison spectra of known compounds and group frequencies, enables one to deduce a considerable amount of structural information from an infra-red spectrum. It should be noted that the complete interpretation of the spectrum can be a very difficult or impossible task. One is usually content to assign the strongest bands and to be able to explain some of the weaker ones as overtones or combinations.

Analysis of the Spectra obtained during the synthesis of the Model FWA.

(a) 2,4-bis-(diethylamino)-6-chloro-s-triazine:

Examination of the spectrum (IR 1) obtained for the product from the synthesis of the above compound yields the following information:

Absorption frequency (cm^{-1})	Possible assignments and inference
no absorption above 3000	no aromatic C-H bonds; no O-H bonds
3000, 2900, 2800	non aromatic C-H bonds
1580	C-N stretching frequency
1500--1400	these bands indicate C-C bonds
1300--900	C-H bending vibrations
800	possibly due to C-Cl

The above are consistent with the spectrum expected for 2,4-bis-(diethylamino)-6-chloro-s-triazine.

(b) 4-Nitro toluene

Examination of the spectrum (IR 2) for the above compound yields the following information:

Absorption frequency cm^{-1}	Possible assignment and inference
3150 and 3100	aromatic C-H bonds
2850 and 2950	non aromatic C-H bonds
1600	aromatic ring stretching frequency
1520 and 1350	N-O stretching
1300--900	aromatic C-H and C-C bonds
740	possibly due to the two pair of adjacent H's of a p-disubstituted aromatic ring

The above results are consistent with the spectrum expected for 4-nitro toluene.

(c) 2-Methyl-5-nitro-benzenesulphonic acid potassium salt:

Examination of the spectrum (IR 3) obtained for the product of the synthesis of the above compound yields the following information:

Absorption frequency cm^{-1}	Possible assignment and inference
3125 and 3100	aromatic C-H bonds
3000 and2950	non aromatic C-H bonds
1615	aromatic ring stretching frequency
1540 and 1360	N-O stretching
1200	S=O stretching
1300--900	various bands from the aromatic ring C-C and C-C stretches: the pattern is more complex than that for compound (b) caused by the addition of the SO_3 group
740	possibly the C-H stretching frequency of the pair of adjacent H's on the ring

The above results are consistent with the spectrum expected for the potassium salt of 2-methyl-5-nitro-benzenesulphonic acid.

(d) 2-Carboxy-5-nitro-benzenesulphonic acid potassium salt.

Examination of the spectrum (IR 4) obtained for the product from the synthesis of the above compound yields the following information:

Absorption frequency cm^{-1}	Possible assignment and inference
3500 and 3400	could be O-H of the acid but the broad bands at 2350 and 1900 indicate that the sample is wet; this absorption is probably due to O-H in water
3125 and 3100	aromatic C-H

Absorption frequency cm^1	Possible assignment and inference
1725	C=O bond: the frequency for aromatic carboxylic acids is usually 1680-1690. The frequency may be high because of formation of the anhydride with the adjacent SO_3 group.
1600	aromatic ring stretching frequency
1540 and 1310	N-O stretching frequency
1210	S-O stretching frequency
1300--800	complex pattern of bands possibly due to the aromatic ring C-H and C-C stretching
740	possibly the C-H stretching of the pair of adjacent H's on the ring

The above are not quite consistent with the spectrum expected for 2-carboxy-5-nitro-benzenesulphonic acid. There is however, some evidence that the anhydride has been produced, ie the absence of any O-H absorption and the higher than usual C=O absorption frequency.

(e) 5-Amino-2-carboxy-benzenesulphonic acid.

Examination of the spectrum (IR 5) obtained for the product from the synthesis of the above compound yields the following information:

Absorption frequency cm^{-1}	Possible assignment and inference
3200--3100	NH_2 bands
1730	C=O bonds: again higher than normally expected for aromatic carboxylic acids
1600	aromatic ring stretching frequency
1520	N-H stretching
1240	S=O stretching

The large absorption bands for the NO_2 group have completely disappeared indicating that the reduction of the NO_2 to the NH_2 has been complete. The above are not quite consistent with the expected spectrum for 5-amino-2-carboxy-benzenesulphonic acid but indicate that the anhydride may have been formed.

(f) 2,4-(diethylamino)-6-(5-amino-2-carboxy-benzenesulphonic acid)-s-triazine.

Examination of the spectrum (IR 6) obtained for the product from the synthesis of the above compound yields the following information:

Absorption frequency cm^{-1}	Possible assignment and inference
broad medium peaks above 3000	aromatic C-H and N-H
3000 and 2950	non aromatic C-H
1740	C=O; again at a higher than normal frequency for aromatic carboxylic acids
1600	aromatic ring stretching
1220	S=O stretching

The above are not quite consistent with the spectrum expected for 2,4-bis-(diethylamino)-6-(5-amino-2-carboxy-benzenesulphonic acid)-s-triazine but indicate that the anhydride has been formed.

(g) 2,4-Bis-(diethylamino)-6-(4-amino-benzoic acid)-s-triazine.

Examination of the spectrum (IR 7) obtained for the product from the synthesis of the above compound yields the following information:

Absorption frequency cm^{-1}	Possible assignment and inference
3450	O-H
3350	N-H
3200 and 3050	aromatic C-H
3000 and 2950	non aromatic C-H

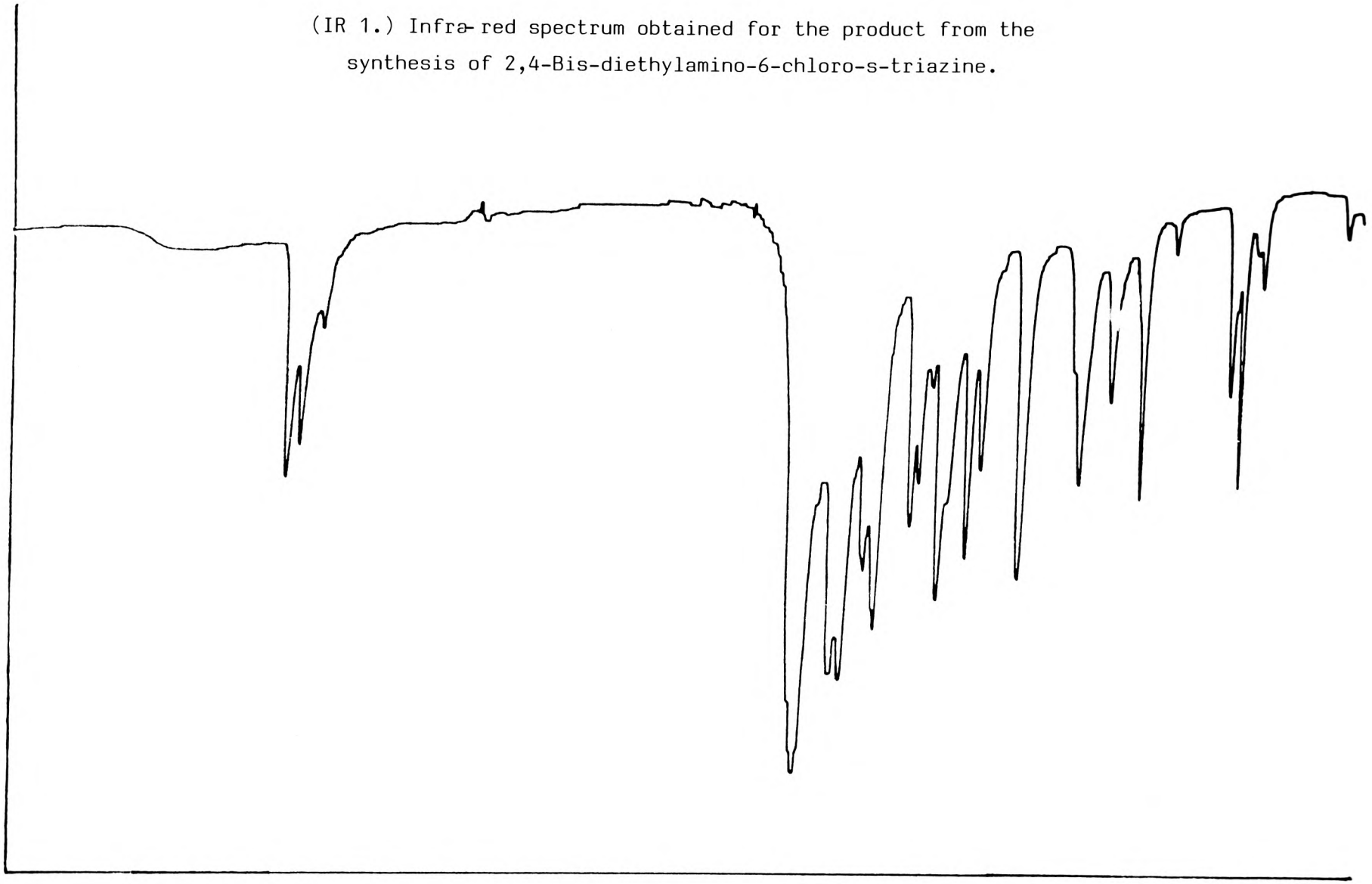
Absorption frequency cm^{-1}	Possible assignment and inference
1690	C=O at a more normal frequency
1600	aromatic ring stretching
above and below 1600	C-N
1400--1200	C-H and C-C

The above are consistent with the spectrum expected for 2,4-bis-(diethylamino)-6-(4-amino-benzoic acid)-s-triazine.

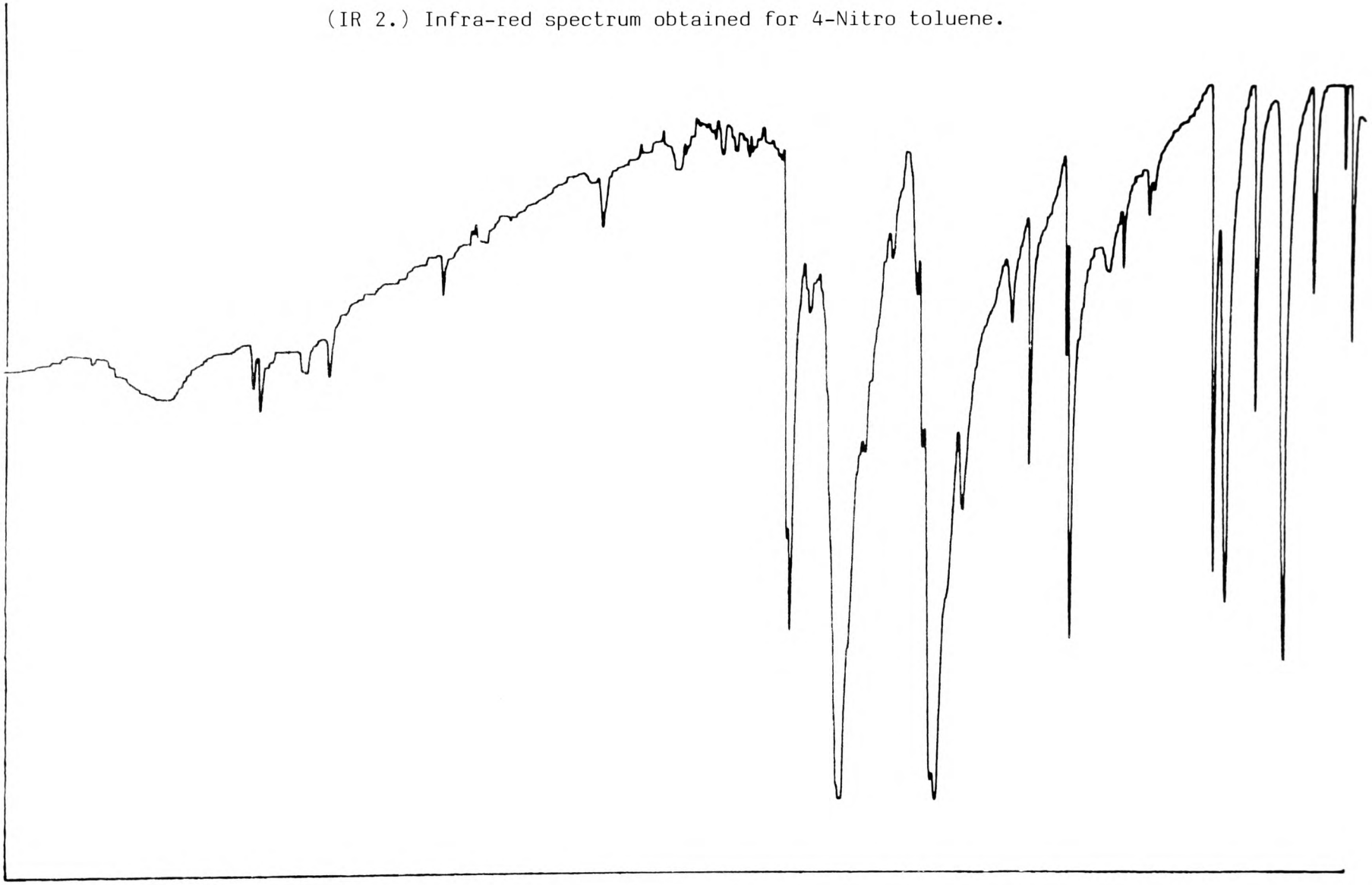
Discussion:

As previously mentioned it is not normally possible to interpret the spectrum of a complex molecule to determine exactly the compound under investigation. It was possible to examine and assign many major groups in the above spectra. On the evidence it would appear that all the compounds synthesised are the desired ones with the exception of those containing both CO_2H and SO_3H. It would seem, but with limited evidence, that these compounds were synthesised as the anhydride. This corresponds to the fact that commercially available compounds containing these two groups adjacent to each other are supplied as the anhydride, which seems to be their most stable form. However, it should be noted that other evidence must be obtained before the presence of the anhydride is confirmed.

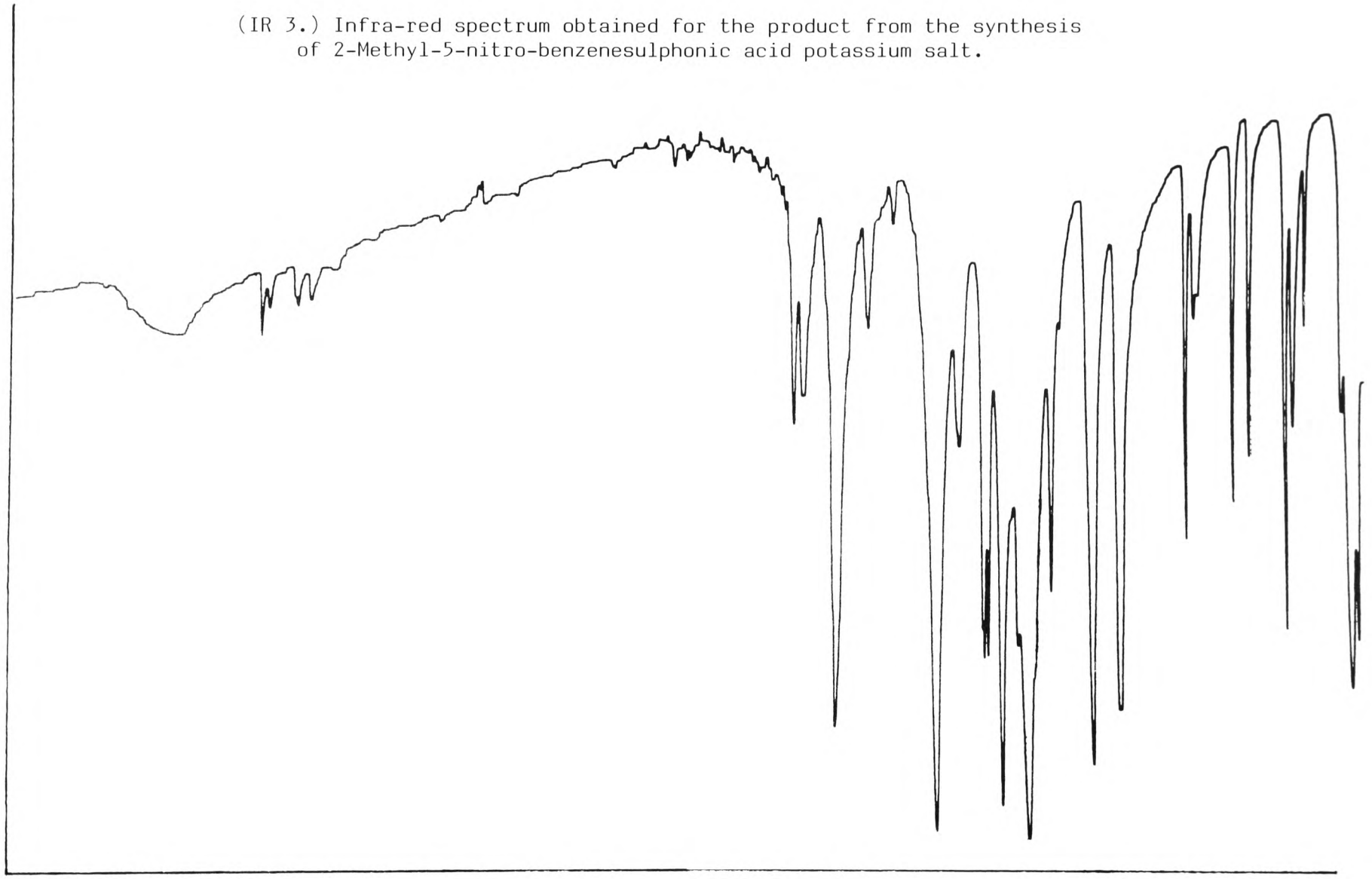
(IR 1.) Infra-red spectrum obtained for the product from the synthesis of 2,4-Bis-diethylamino-6-chloro-s-triazine.



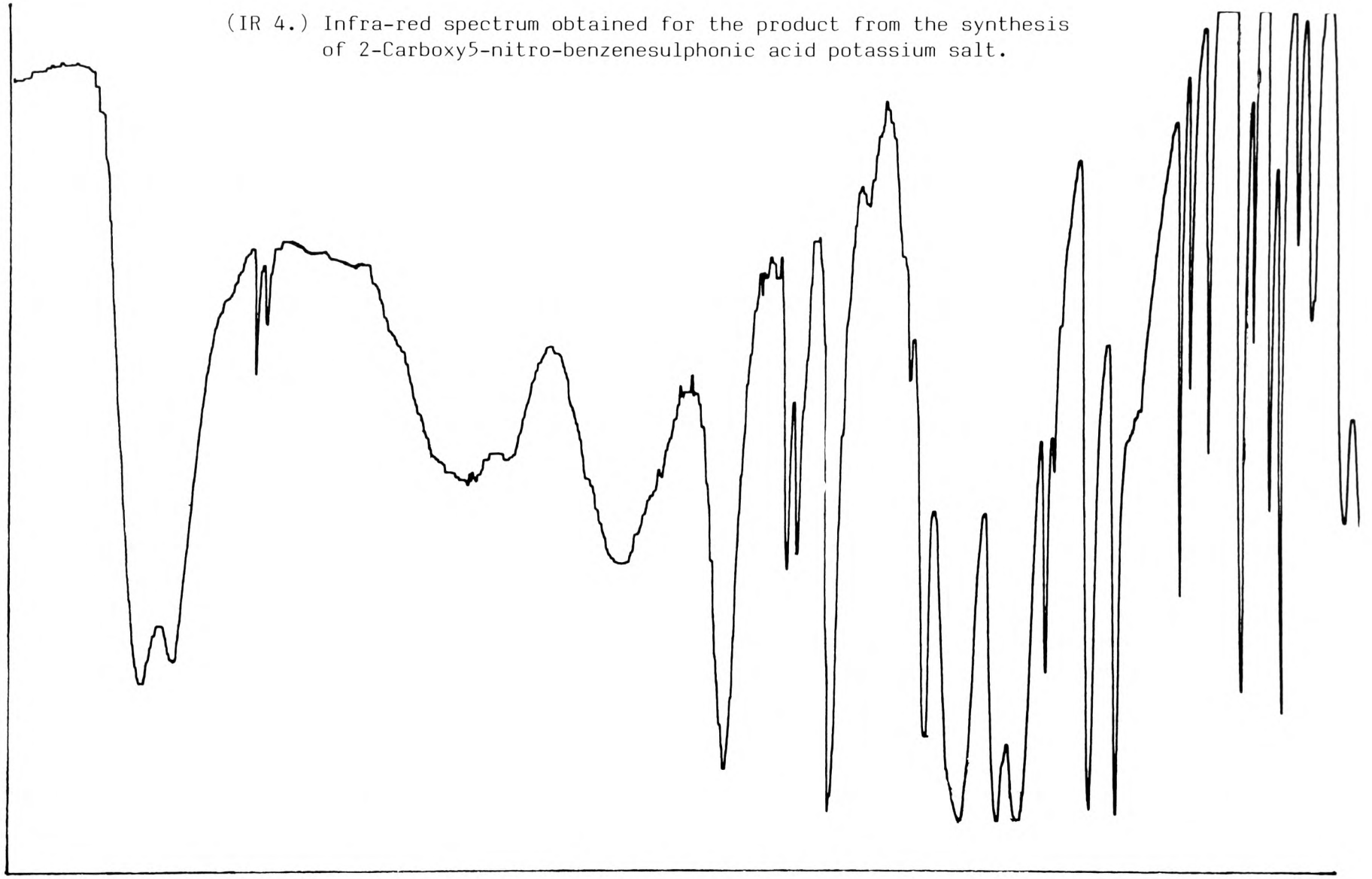
(IR 2.) Infra-red spectrum obtained for 4-Nitro toluene.



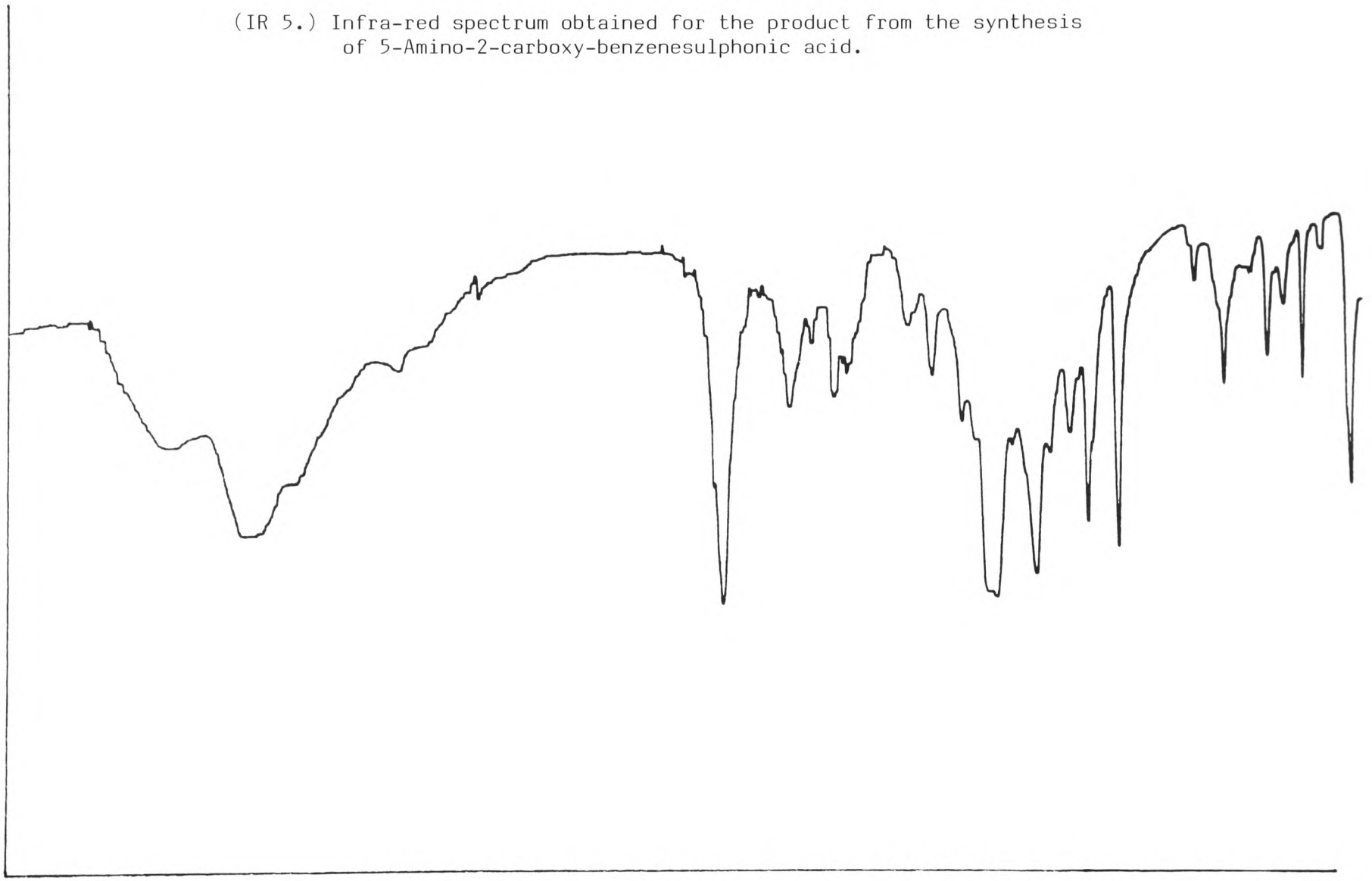
(IR 3.) Infra-red spectrum obtained for the product from the synthesis of 2-Methyl-5-nitro-benzenesulphonic acid potassium salt.



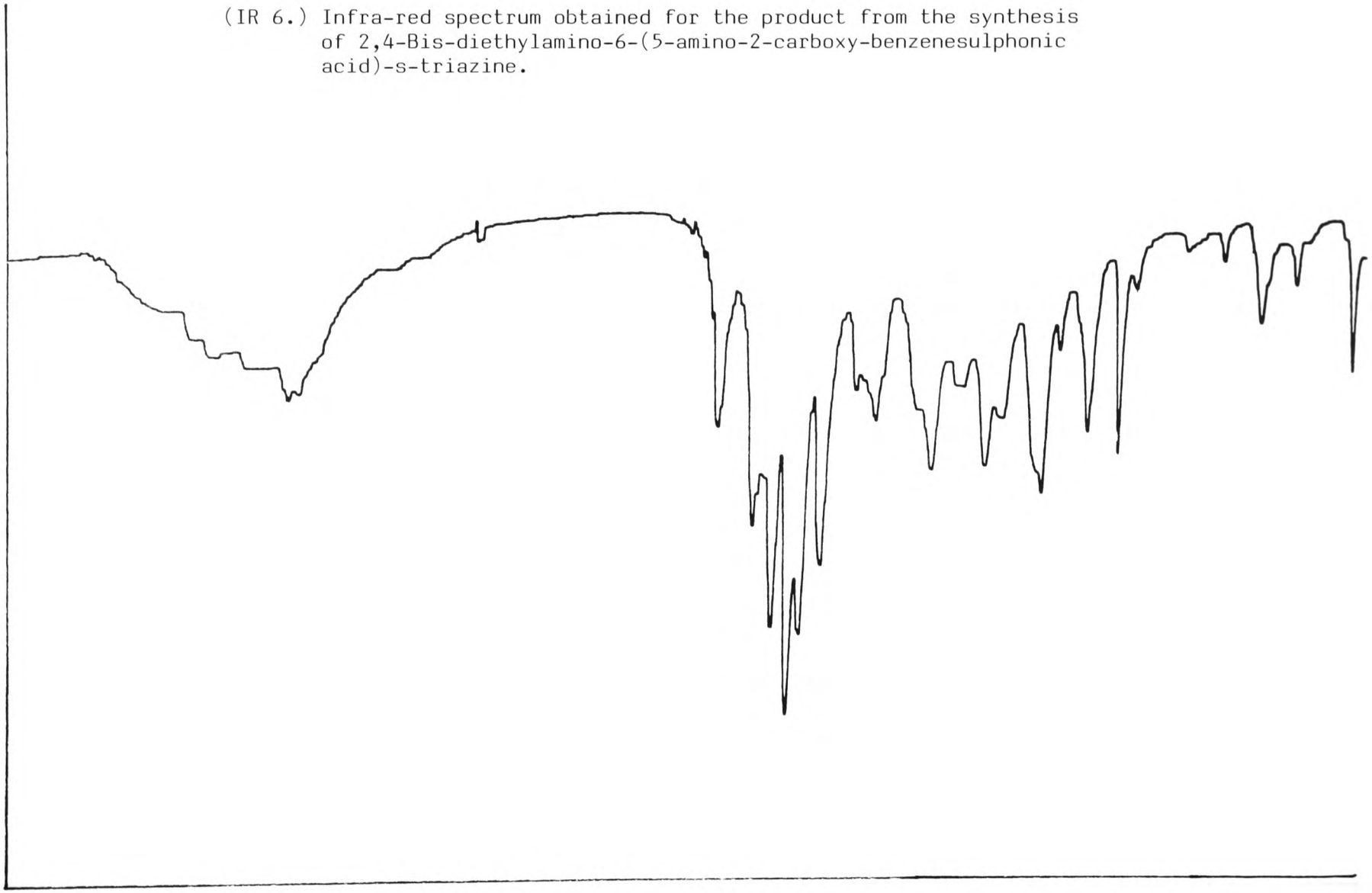
(IR 4.) Infra-red spectrum obtained for the product from the synthesis of 2-Carboxy5-nitro-benzenesulphonic acid potassium salt.



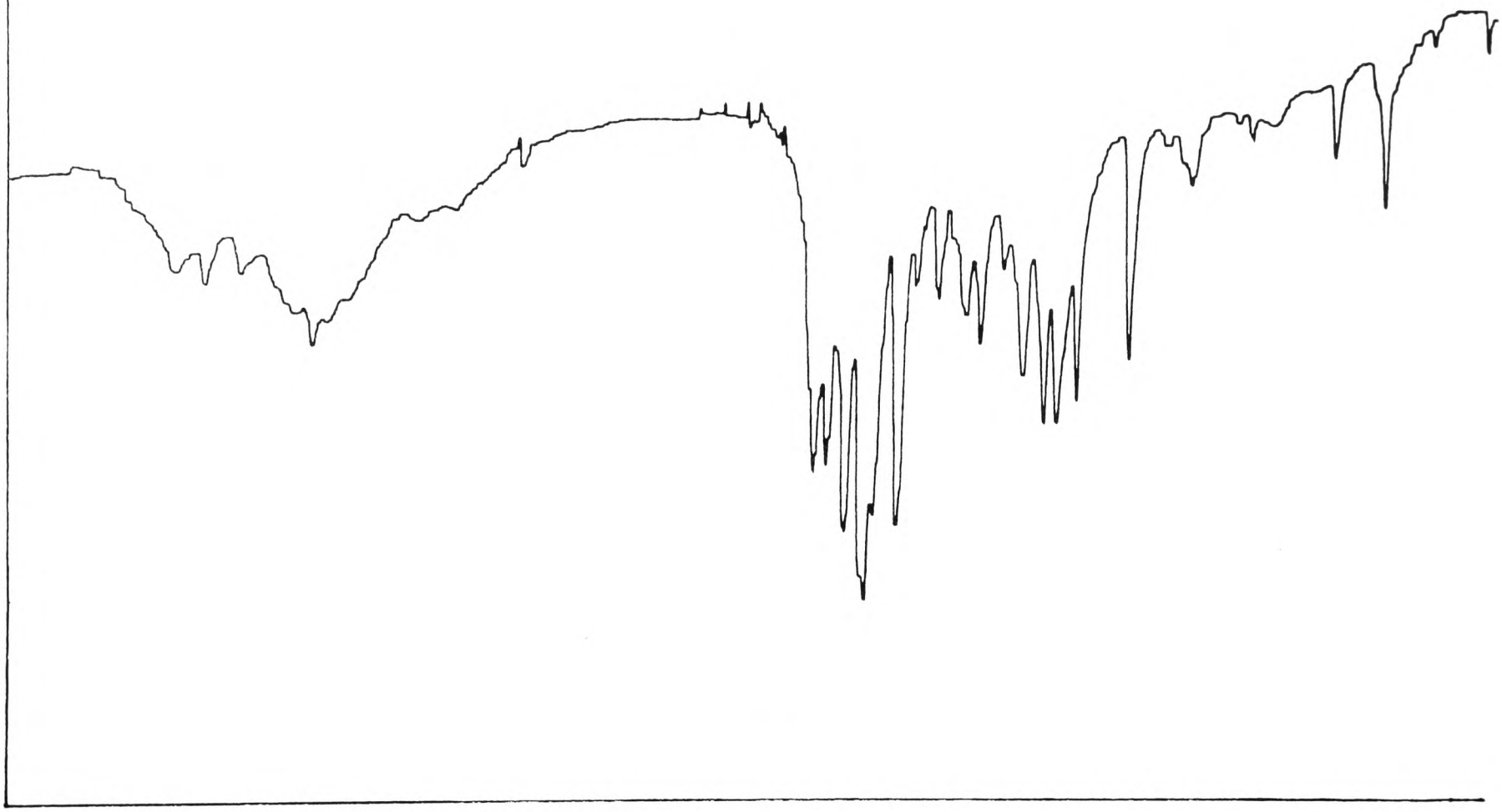
(IR 5.) Infra-red spectrum obtained for the product from the synthesis of 5-Amino-2-carboxy-benzenesulphonic acid.



(IR 6.) Infra-red spectrum obtained for the product from the synthesis of 2,4-Bis-diethylamino-6-(5-amino-2-carboxy-benzenesulphonic acid)-s-triazine.



(IR 7.) Infra-red spectrum obtained for the product from the synthesis of 2,4-Bis-diethylamino-6-(4-amino-benzoic acid)-s-triazine.



APPENDIX 4.

N. M. R. SPECTRA.

The N. M. R. Spectra were run by Mr. S Reid, N. M. R. Technician at The Polytechnic of Wales.

Introduction:

It is not intended to give a definitive explanation of nuclear magnetic resonance spectroscopy (N. M. R.): - there are many textbooks available on the subject[64, 65, 66]; but merely to give some background information prior to interpreting some spectra.

Transitions between nuclear spin energy levels give rise to the phenomenon of N. M. R.

The direction of a spin angular momentum vector is conventionally defined such that the spinning body, viewed along the vector, twists in the same sense as a right hand screw, and its magnitude is given by:

$$P = \hbar[I(I+1)]^{1/2}$$

The constant $\hbar$ is $h/2\pi$ where h is known as Planck's constant and I is the spin quantum number; for a proton I = 1/2. Some nuclei have I = 0 making them magnetically inactive, a fact which leads to no signal in the N. M. R. spectrum. Important examples are the major isotopes ^{12}C and ^{16}O making the N. M. R. spectra of organic molecules much simpler.

In principle transitions may be induced between the energy levels (the energy separations between levels is constant) in magnetically active nuclei by the use of electromagnetic radiation of the appropriate wavelength.

The transition for any isolated nuclear isotope occurs at a single frequency since all the energy separations are equal and transitions are allowed only between adjacent levels. Thus the nucleus can interact with radiation whose frequency depends only upon the applied field and the nature of the nucleus.

The resonance frequency can be found by either applying a constant field and varying the frequency of the oscillating field, or by keeping the frequency of the oscillating field fixed and varying the magnetic field. The latter method is the more convenient experimentally and in N. M. R. spectroscopy the energy absorbed by the nucleus is monitored as the magnetic field is increased. When resonance is attained the energy absorbed produces a signal at the detector and this signal is amplified and recorded as a band in the spectrum.

The resonance frequencies of nuclei in a sample are measured relative to the resonance frequency of a nucleus in a reference compound, and the frequencies are quoted relative to the reference frequency. The normal reference compound used for ^1H nuclei in organic compounds is tetramethyl silane (TMS). The protons in TMS are all equivalent and the N. M. R. spectrum consists of a single peak.

The position of the peaks in a spectrum relative to the reference peak can be quoted in terms of the chemical shift, δ, as

$$\delta = (\Delta\nu/\nu) \times 10^6$$

where $\Delta\nu$ is the difference in frequency between the position of a peak and that of the reference and ν is the frequency of the oscillating field. The chemical shift, δ, is dimensionless and is expressed in ppm on account of the factor of 10^6 which is included in the definition in order to avoid having to quote very small values.

Just as in other branches of spectroscopy absorption bands arise at different frequencies. This difference arises because different nuclei in a molecule experience different magnetic fields as a result of the secondary magnetic fields associated with the molecule. These secondary fields arise from the induced circulation of electrons in the molecule under the influence of the applied field. The secondary fields may

either oppose the applied field at a particular nucleus in the molecule or reinforce the applied field; that is the nucleus is either positively shielded or negatively shielded by the secondary field. In the former case the effective field experienced by the nucleus is less than the applied field and the value of the applied field necessary to bring the nucleus into resonance will be greater than if there were no secondary field. Thus when the nucleus is positively shielded the resonance frequency moves upfield (lower δ values). Conversely when the nucleus is negatively shielded the resonance frequency moves downfield (higher δ values).

Hydrogen (^{1}H) Spectra

An important feature of ^{1}H spectra is that, under the correct operating conditions, the area of the bands, i.e. the integrated intensities, are in approximately the same ratio as the number of protons responsible for the resonance. Consequently if the total number of protons in the molecule is known the number of protons in each particular environment can often be found.

The fact that an individual nucleus can give rise to a set of bands in a spectrum arises from the effect of the spin associated with neighbouring nuclei on the field experienced by the nucleus undergoing resonance. The effect of the spin of the neighbouring nuclei on a particular nucleus is transmitted via the electrons in the intervening bonds.

If a proton has N chemically equivalent neighbouring protons then the number of bands in the multiplet is N + 1.

Spin spin coupling is not limited to nuclei of the same type and coupling can, in principle, occur between any two nuclei which have magnetic moments. Coupling between the ^{13}C and the ^{1}H nuclei is not

normally observed in routine ^{1}H spectra as the proton-resonance bands for structures such as $R_3{}^{13}C-{}^{1}H$ are very weak on account of the low relative abundance (1.1%) of the ^{13}C nucleus.

Carbon (^{13}C) Spectra.

Formerly the study of the ^{13}C nucleus was difficult since it gives rise to extremely weak signals but the introduction of Fourier Transform methods caused a big upsurge in the study of this nucleus.

The phenomena of chemical shift and spin spin coupling are observed in the spectra of ^{13}C molecules and generally both are considerably larger than for their hydrogen counterparts. Thus, while a range of some 15ppm contains virtually all the known hydrogen chemical shifts, the chemical shifts for ^{13}C nuclei covers a range of several hundred ppm.

Coupling between ^{13}C and ^{13}C does not normally occur as the chance of more than one ^{13}C being in a molecule is extremely high. C-H coupling can be removed by simultaneously applying a strong radio frequency field at the resonance frequency of the protons.

Analysis of the spectra obtained during the synthesis of the model FWA.

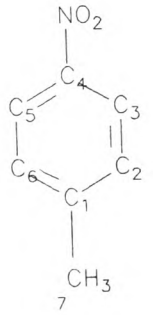
(a) 2,4-bis-(diethylamino)-6-chloro-s-triazine:

Examination of the ^{1}H spectrum (NMR 1) obtained for the product from the synthesis of the above compound shows, apart from the TMS reference peak, only two sets of multiplet peaks. There is a triplet centred at 1.2ppm and a quartet centred at 5.35ppm. The triplet represents CH_3 protons coupled to each of the CH_2 protons whilst the quartet represents the CH_2 protons coupled with each of the three CH_3 protons. The ratio of the relative intensities of the multiplets CH_3: CH_2 is approximately 3:2 indicating that there are one and a half times as many protons in a Ch_3 environment as protons in a CH_2 environment.

The spectrum is consistent with that expected for 2,4-bis-(diethylamino)-6-chloro-s-triazine.

Examination of the ^{13}C spectrum (NMR 2) for the above compound shows, apart from the TMS reference and the triplet for the $CDCl_3$ solvent, only four single peaks. The peaks are at 13, 41.5, 164, and 169ppm and are consistent with the peaks expected for CH_3, CH_2, N–C–N, and N–C–Cl respectively.

(b) 4-Nitro toluene:



^1H Spectrum:

Examination of the structure of 4-nitro toluene shows that there are three types of proton; the protons attached to carbon atoms 3 and 5, 2 and 6, and the CH_3 protons on carbon 7. The protons on carbons 3 and 5 are adjacent to a carbon with an electron withdrawing group attached which would push the normal resonance frequency of a benzene proton (7.37ppm) downfield to a higher δ value. The protons on carbons 2 and 6 are adjacent to a carbon with an electron releasing group attached which would push the normal resonance frequency upfield to a lower δ value. The CH_3 protons usually have a resonance frequency at much lower δ values than those of the aromatic ring hydrogens.

Examination of the spectrum of 4-nitro toluene (NMR 3) shows that there is a singlet at 2.42ppm (CH_3 protons), a doublet centred at 7.27ppm

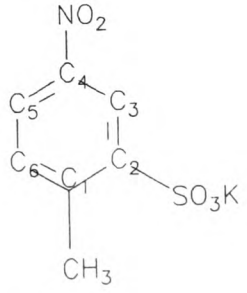
(protons on carbons 2 and 6), and a doublet centred at 8.04ppm (protons on carbons 3 and 5). The doublets are typical of the coupling pattern for an aromatic AA,BB structure and the spectrum is consistent with that expected for 4-nitro toluene.

^{13}C spectrum:

Examination of the structure of 4-nitro toluene shows that there are five types of carbons; carbons 3 and 5: 2 and 6: 1: 4: and 7. The carbon attached to the NO_2 group does not always show up in the spectrum of a compound because the nitro group broadens it by reducing the relaxation time T_2. As in the case of the proton spectrum, the carbons adjacent to the carbon with the nitro group attached have a resonance frequency downfield of the normal resonance frequency of benzene carbons, while those adjacent to the carbon attached to the methyl group have a resonance frequency upfield of the normal frequency. Quaternary carbons (1 and 4) are normally pushed downfield of their usual frequencies mainly due to the absence of attached protons.

Examination of the spectrum of 4-nitro toluene (NMR 4) shows four single peaks at 21.45, 123.41, 129.84, and 146.17ppm which can be assigned as carbons 7; 2 and 6; 3 and 5; and 1 respectively. The peak for carbon 4 is absent. This is consistent with the spectrum expected for 4-nitro toluene.

(c) 2-Methyl-5-nitro benzenesulphonic acid potassium salt:



7

^{1}H spectrum:

Examination of the above structure shows that there are four types of protons; those attached to carbon atoms 3; 5; 6; and 7. One would expect a doublet for the proton attached to carbon 3 because of meta coupling with the proton on carbon 5, and a doublet for the proton attached to carbon 6 because of ortho coupling with the proton on carbon 5. One would predict a pair of doublets for the proton attached to carbon 5 due to ortho coupling with the proton attached to carbon 6, and meta coupling with the proton attached to carbon 3. One would expect a single peak at much lower δ value for the protons attached to carbon 7. Since the proton on carbon 3 is adjacent to two electron withdrawing groups one would expect it to have the greatest chemical shift. the proton attached to carbon 5 is adjacent to one electron withdrawing group and so should have the next highest chemical shift. The proton attached to carbon 6 is adjacent to an electron releasing group and we would expect it to have a lower chemical shift.

Examination of the spectrum (NMR 5) for the product from the synthesis of the above compound shows that it is consistent with that expected.

^{13}C Spectrum:

Examination of the structure of the above compound shows that there are seven types of carbon atoms; the six ring carbons and the methyl carbon. One would expect carbon 6 to be the furthest downfield of the ring carbons followed by carbons 2 and 4. Of the three carbons with hydrogens attached, carbon 3 should be furthest downfield followed by carbon 5 then carbon 6. The methyl carbon 7 should be much further upfield than the ring carbons.

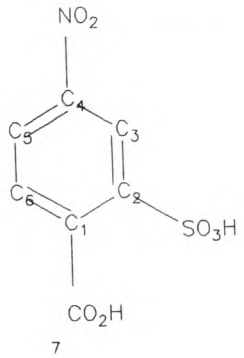
Examination of the spectrum (NMR 6) obtained for the product from the

synthesis of the above compound shows that it is consistent with that expected for 2-methyl-5-nitro-benzenesulphonic acid potassium salt.

(d) 2-Carboxy-5-nitro-benzenesulphonic acid potassium salt:

^{1}H Spectrum:

Examination of the structure of the above compound shows that there are four types of protons, an acid proton on carbon 7; and three ring protons on carbons 3, 5, and 6. The ring protons should show a similar pattern to that of compound (c) with slightly different δ values due to changing the methyl to a carboxy group. The methyl proton peak should disappear with the introduction of an acid proton peak at fairly high δ value.



7

Examination of the spectrum (NMR 7) obtained for the product from the synthesis of the above compound shows that there are only three types of protons and their coupling pattern is consistent with that expected for the ring protons in the above compound. The methyl protons have disappeared as expected but an acid proton peak has not appeared. This may indicate that the anhydride has been formed and supports the data obtained from the I.R. spectrum.

^{13}C Spectrum:

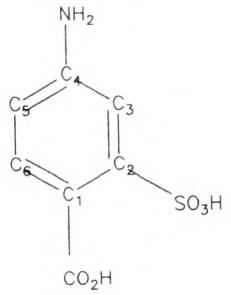
One would expect seven peaks; six aromatic ring carbons and a C=O carbon. One would expect the methyl peak to disappear and a carbonyl peak to appear at a very high δ value.

Examination of the spectrum (NMR 8) obtained for the product from the synthesis of the above compound shows that it is consistent with that expected for the anhydride of that compound.

(e) 5-Amino-2-carboxy- benzenesulphonic acid:

^{1}H Spectrum:

Examination of the structure of the above compound shows that there are six types of protons; three ring protons attached to carbons 3, 5, and 6; two acid protons $-CO_2H$ and SO_3H; and the NH_2 protons. One would expect the coupling pattern of the ring protons to be similar to compound (d) but the effect of replacing the electron withdrawing nitro group with the electron releasing amino group would be to change the relative chemical shifts of the multiplets.



7

Examination of the spectrum (NMR 9) obtained for the product from the synthesis of the above compound shows that there are only four types of protons. The coupling pattern for the three ring protons is as expected and the NH_2 peak can be seen underneath the solvent peaks at approximately 5.5ppm. There are no acid peaks suggesting, as does the I.R. spectrum, that the anhydride has been formed.

^{13}C Spectrum;

One would expect the spectrum of the above compound to be similar to that obtained for compound (d) with the relative shifts being changed by

replacement of an electron withdrawing nitro group with an electron releasing group.

Examination of the spectrum (NMR 10) obtained for the product from the synthesis of the above compound shows the expected seven peaks at the expected chemical shifts. The spectrum is consistent with that for the above compound or the anhydride.

(f) 2,4-bis-(diethylamino)-6-(5-amino-2-carboxy-benzenesulphonic acid)-s-triazine:

^{1}H Spectrum:

Examination of the spectrum (NMR 11) obtained for the product from the synthesis of the above compound shows all the peaks expected, except the acid protons, at the predicted chemical shifts (discussed in the previous pages) and this suggests that the anhydride of the above compound has been formed.

^{13}C Spectrum:

Examination of the spectrum (NMR 12) obtained for the product from the synthesis of the above compound shows that there are eleven peaks. The number, and chemical shifts, are consistent with that predicted by the preceding discussion for either the acid or the anhydride.

(g) 2,4-Bis-(diethylamino)-6-(4-amino-benzoic acid)-s-triazine:

^{1}H Spectrum:

With the removal of the sulphonic acid group the possibility of anhydride formation is eliminated. The spectrum (NMR 13) obtained for the product from the synthesis of the above compound is of average resolution but still shows all the expected peaks and is consistent with that expected for the above compound.

^{13}C Spectrum:

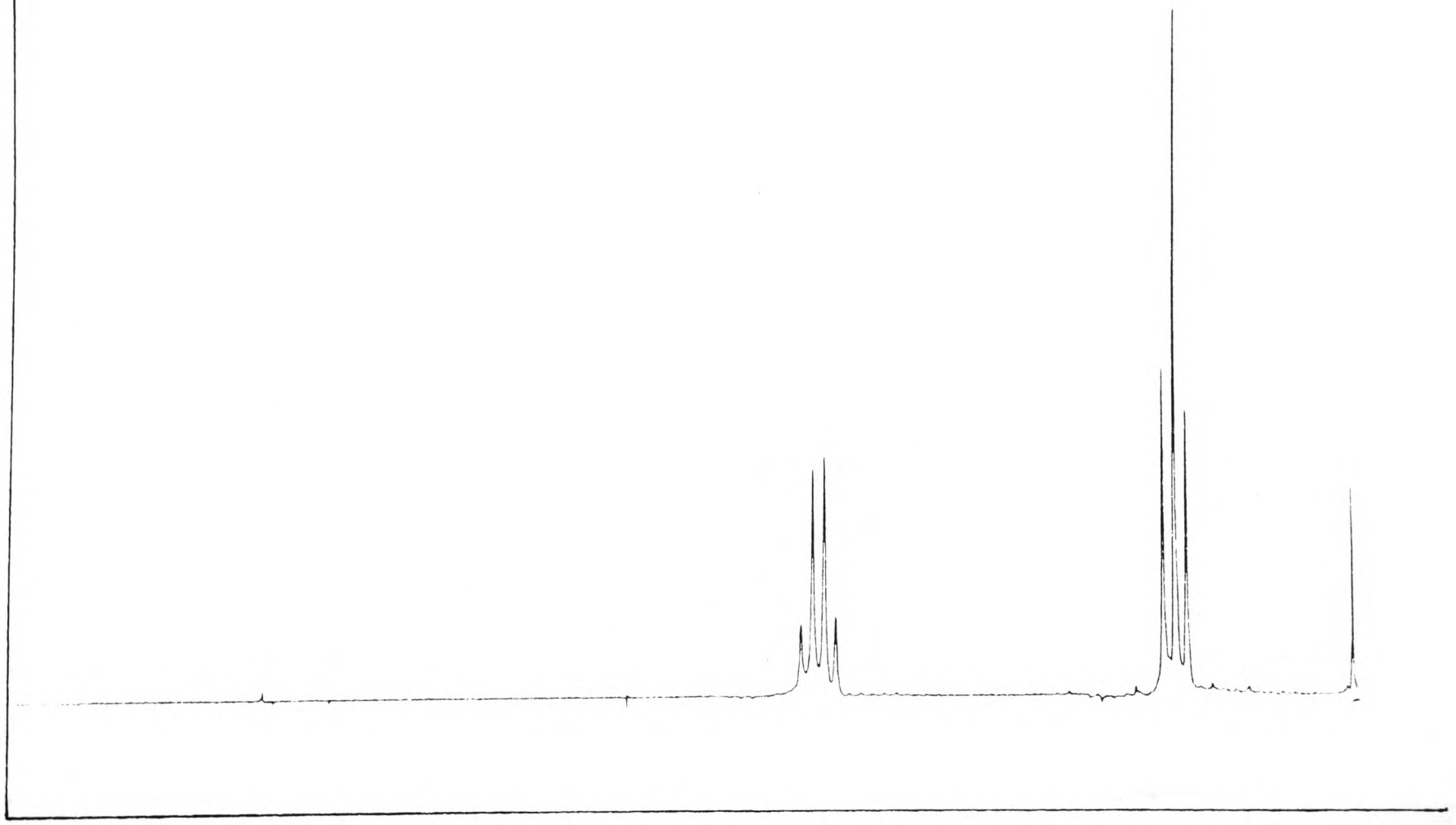
The spectrum (NMR 14) obtained for the product from the synthesis of the

above compound is consistent with that predicted, in preceding discussions for 2,4-bis-(diethylamino)-6-(4-amino-benzoic acid)-s-triazine.

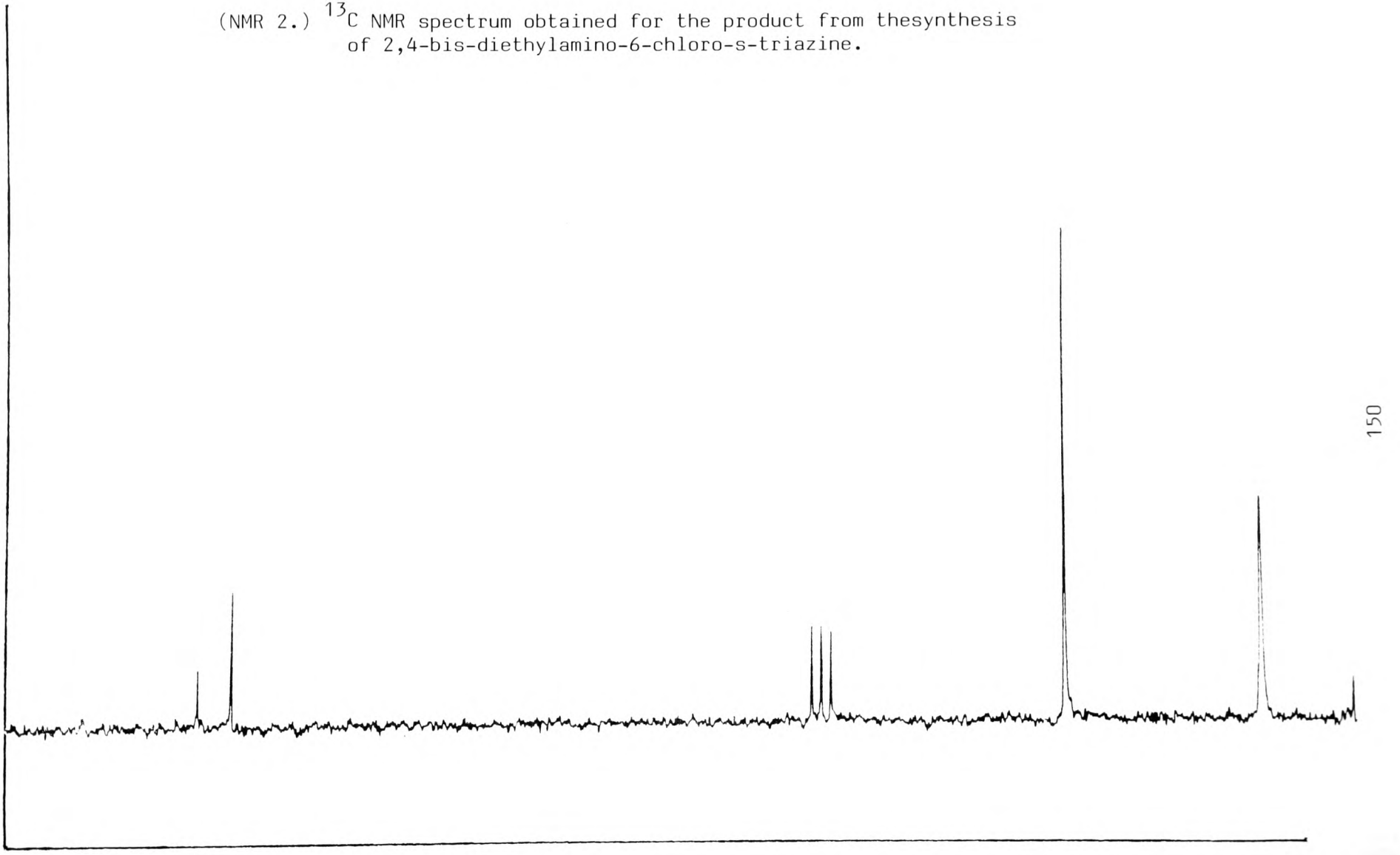
Discussion:

The interpretation of the N.M.R. spectra reinforces the information obtained from the IR spectra. All the compounds synthesised, except those containing both SO_3H and CO_2H were synthesised correctly. Those compounds containing both the acid groups were synthesised as their anhydrides which are the most stable form of the molecules.

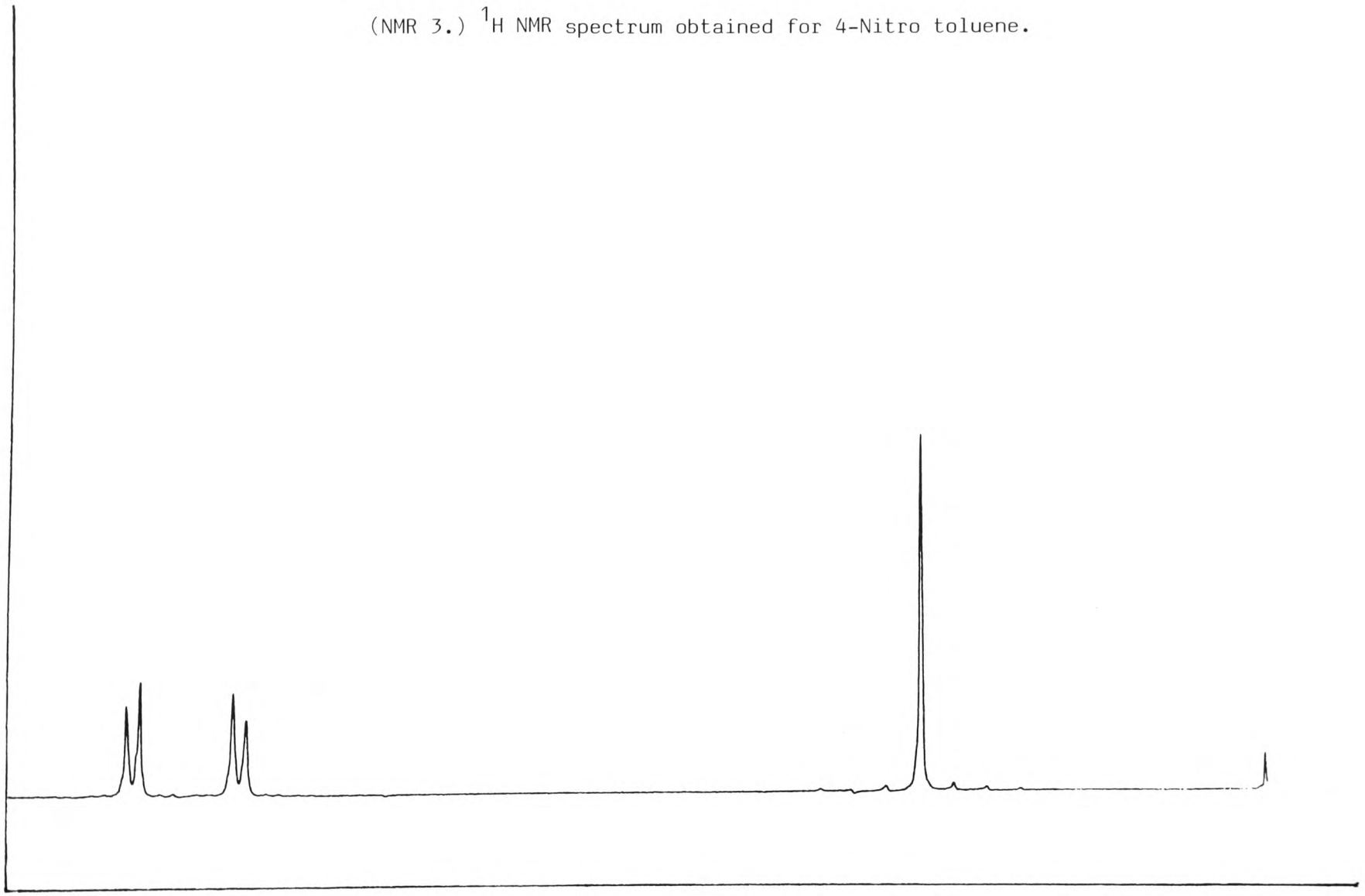
(NMR 1.) ^{1}H NMR spectrum obtained for the product from the synthesis f 2,4-bis-diethylamino-6-chloro-s-triazine.



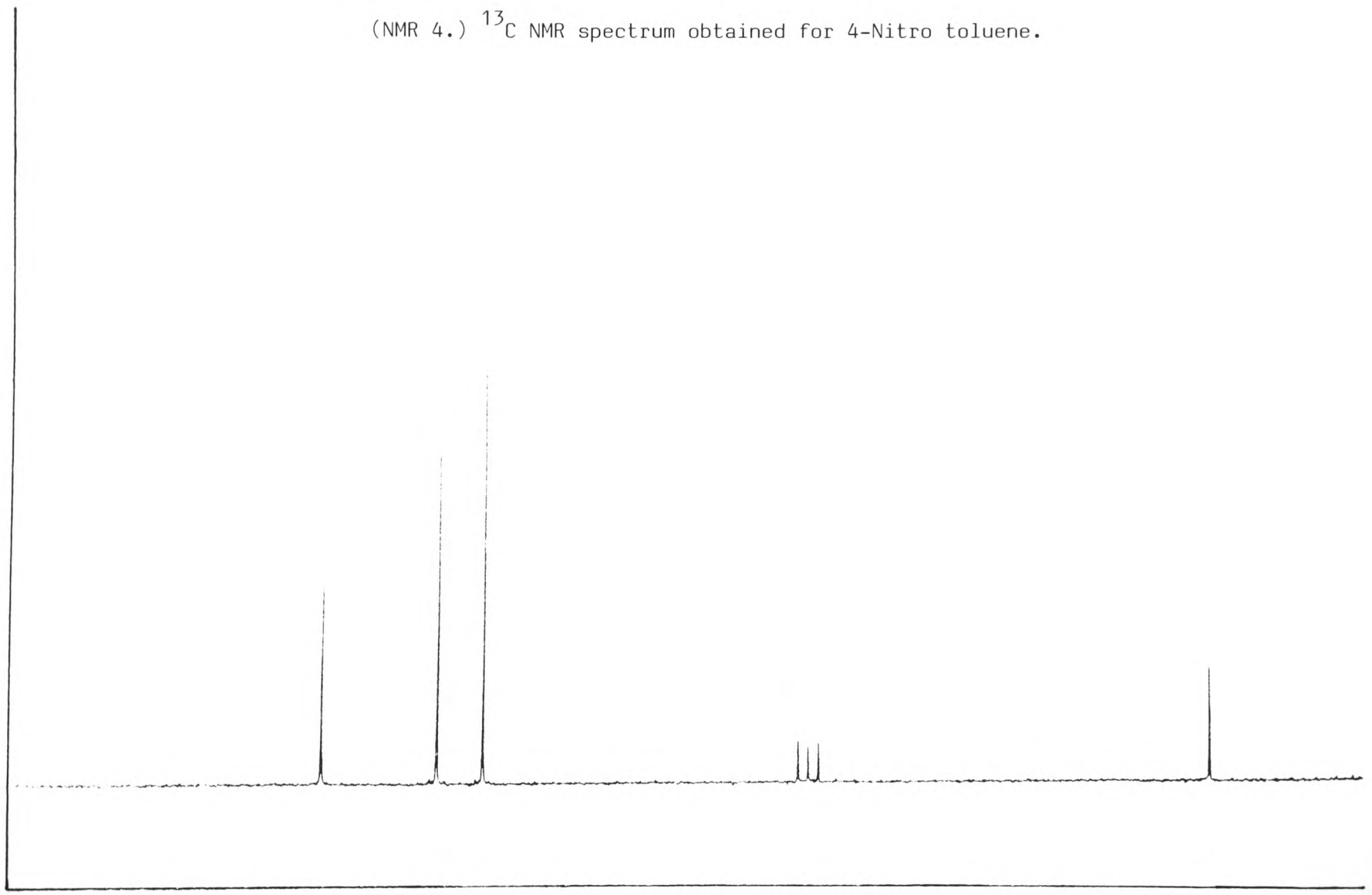
(NMR 2.) ^{13}C NMR spectrum obtained for the product from thesynthesis of 2,4-bis-diethylamino-6-chloro-s-triazine.



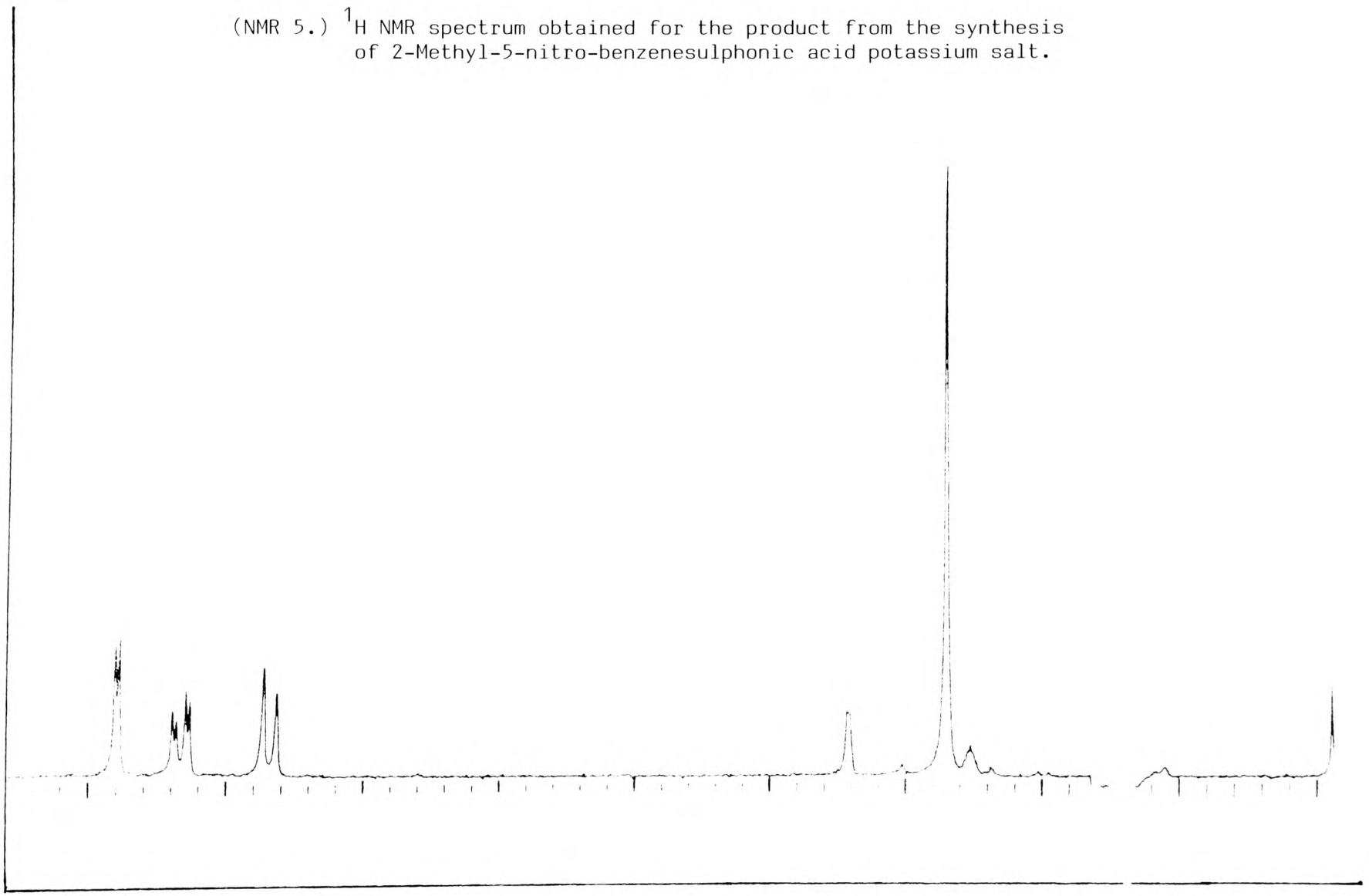
(NMR 3.) ^{1}H NMR spectrum obtained for 4-Nitro toluene.



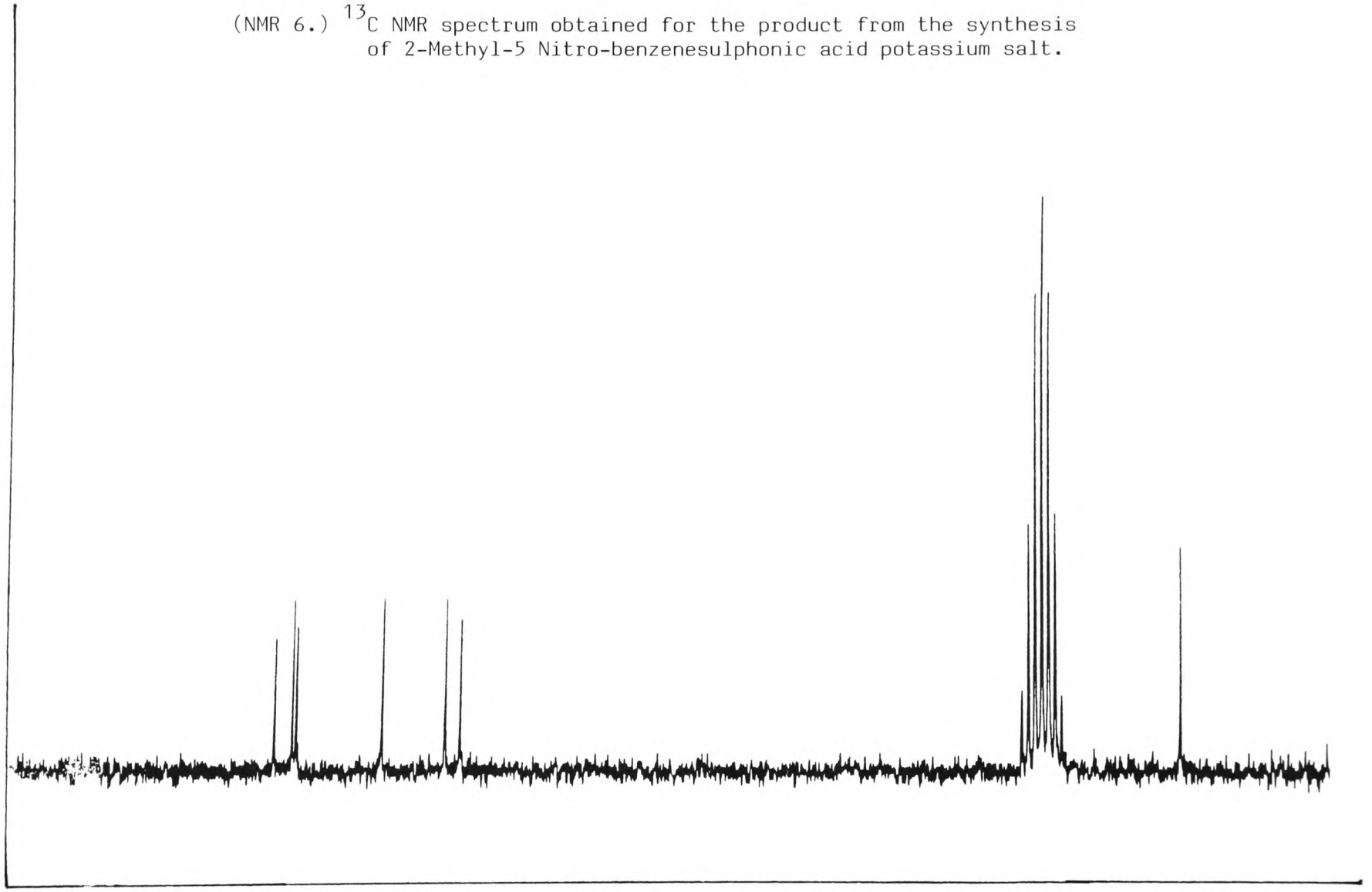
(NMR 4.) ^{13}C NMR spectrum obtained for 4-Nitro toluene.



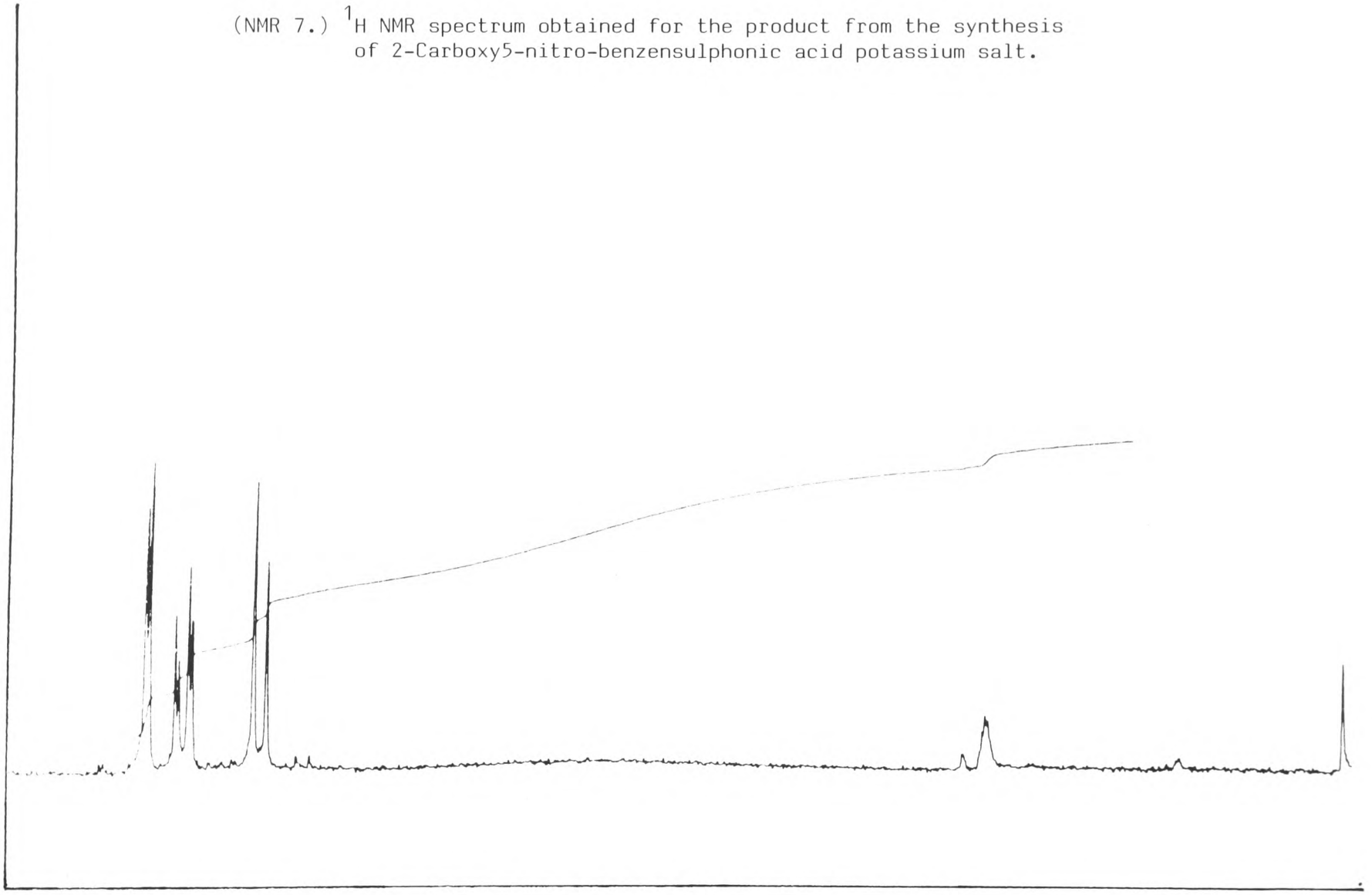
(NMR 5.) ^{1}H NMR spectrum obtained for the product from the synthesis of 2-Methyl-5-nitro-benzenesulphonic acid potassium salt.



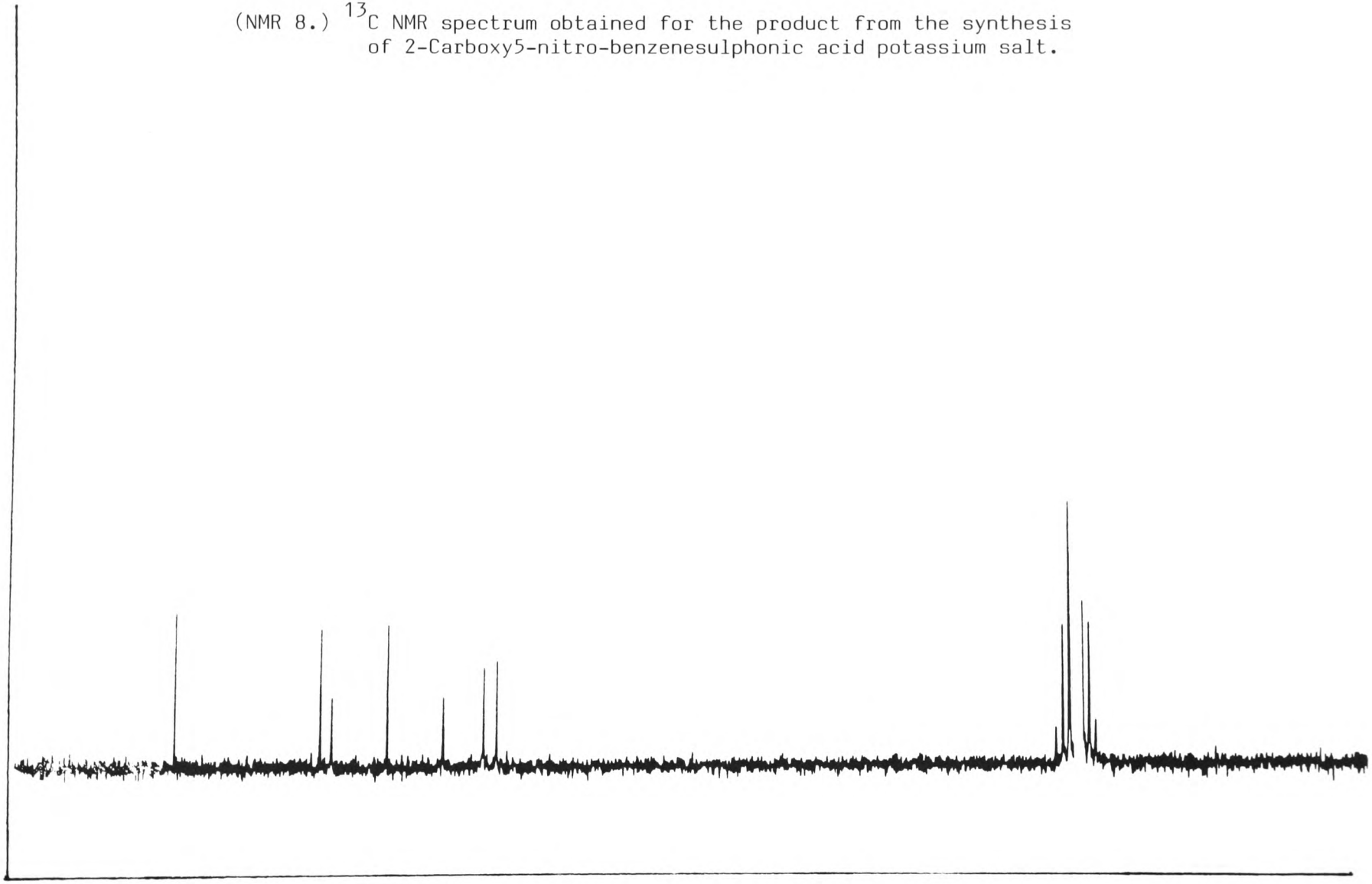
(NMR 6.) ^{13}C NMR spectrum obtained for the product from the synthesis of 2-Methyl-5 Nitro-benzenesulphonic acid potassium salt.



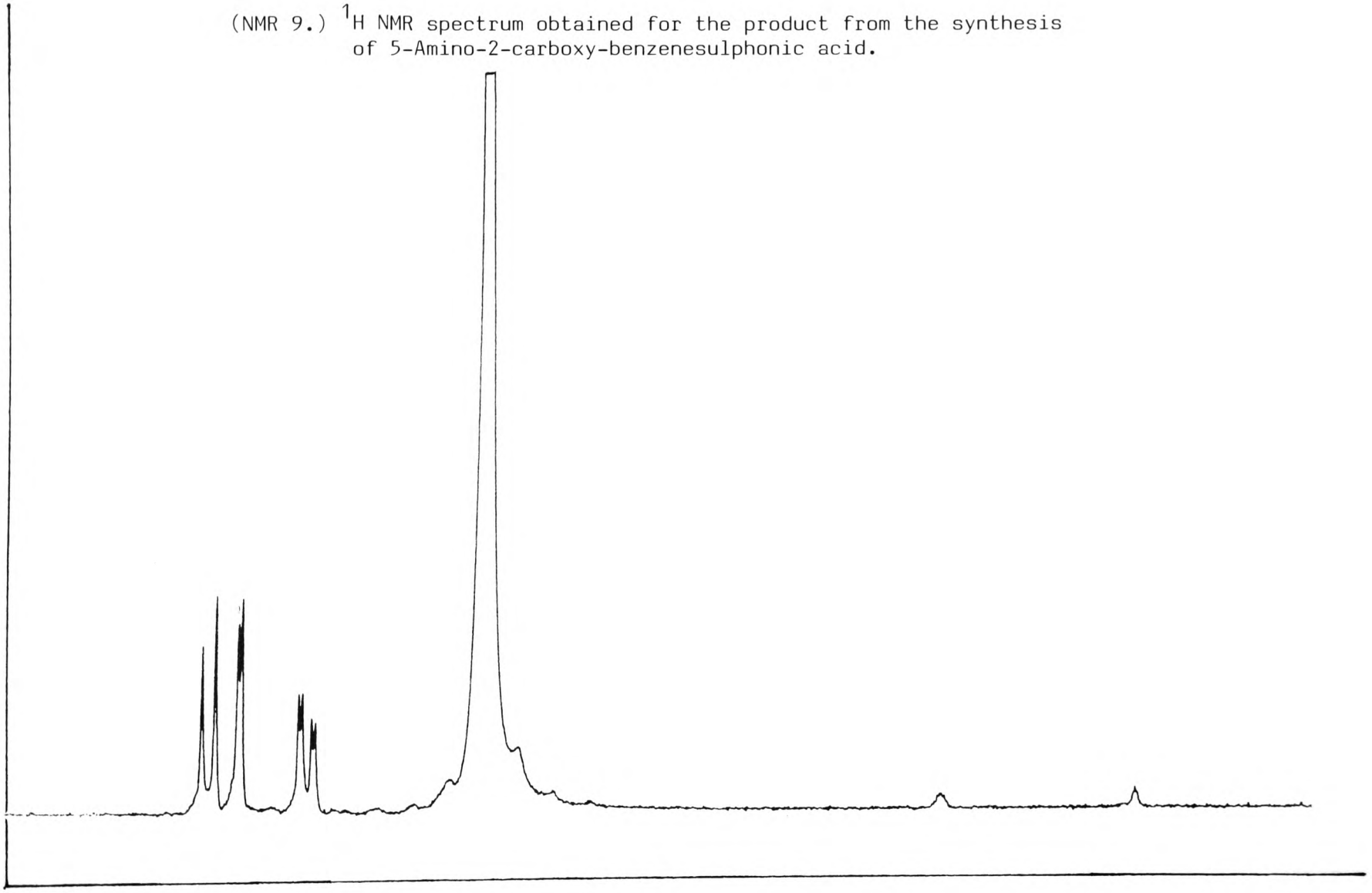
(NMR 7.) ^{1}H NMR spectrum obtained for the product from the synthesis of 2-Carboxy5-nitro-benzensulphonic acid potassium salt.



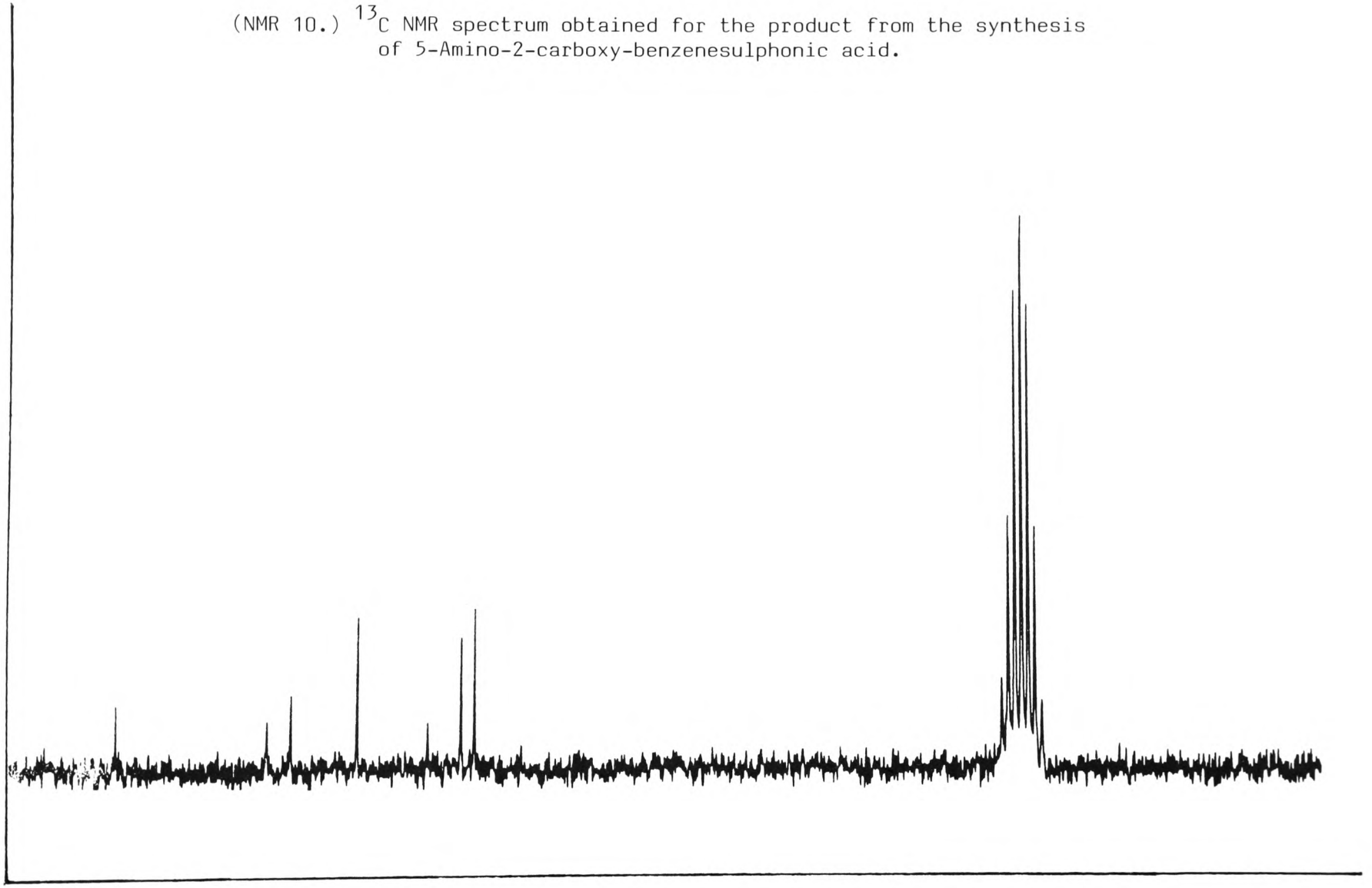
(NMR 8.) ^{13}C NMR spectrum obtained for the product from the synthesis of 2-Carboxy5-nitro-benzenesulphonic acid potassium salt.



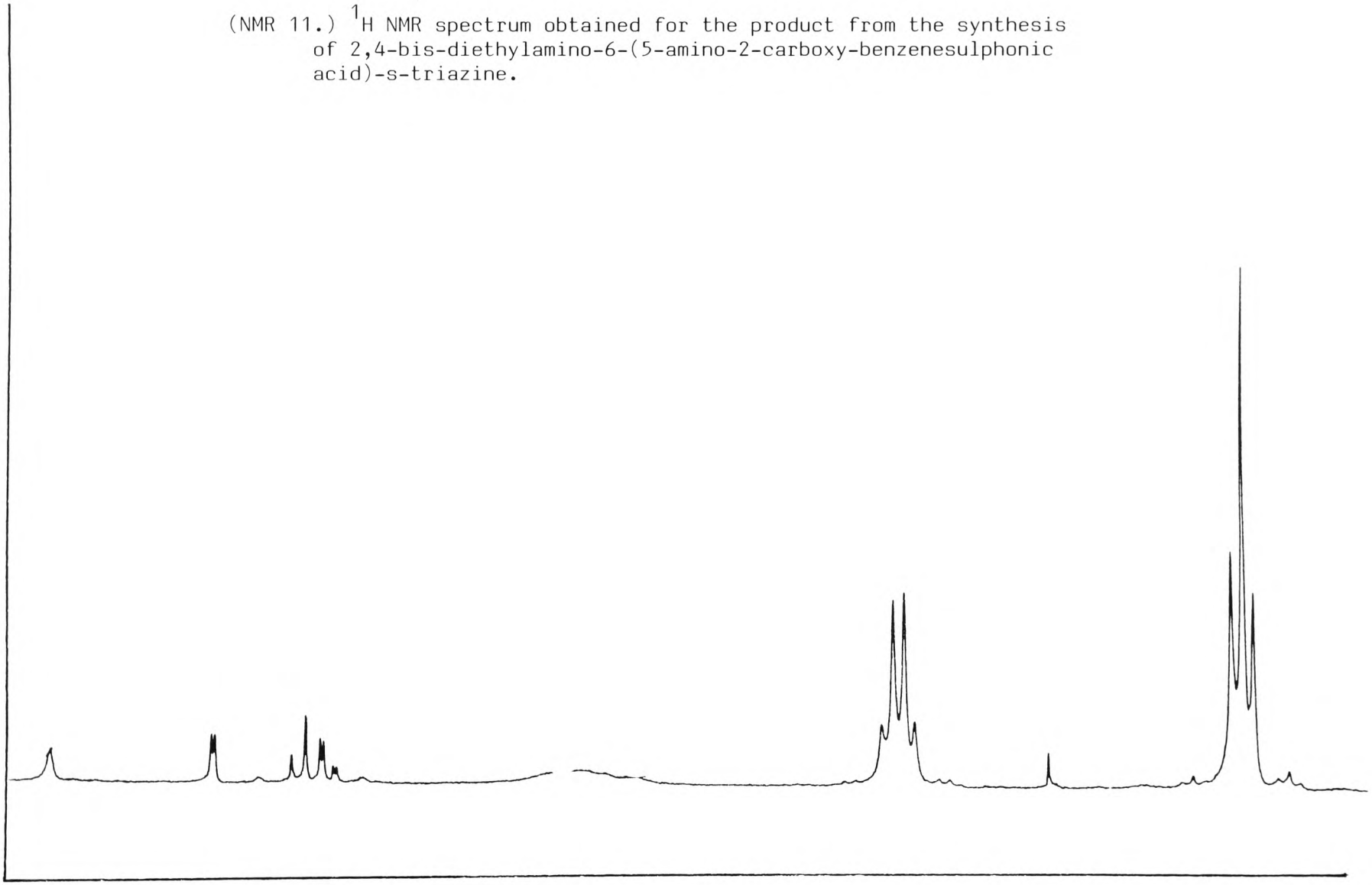
(NMR 9.) ^{1}H NMR spectrum obtained for the product from the synthesis of 5-Amino-2-carboxy-benzenesulphonic acid.



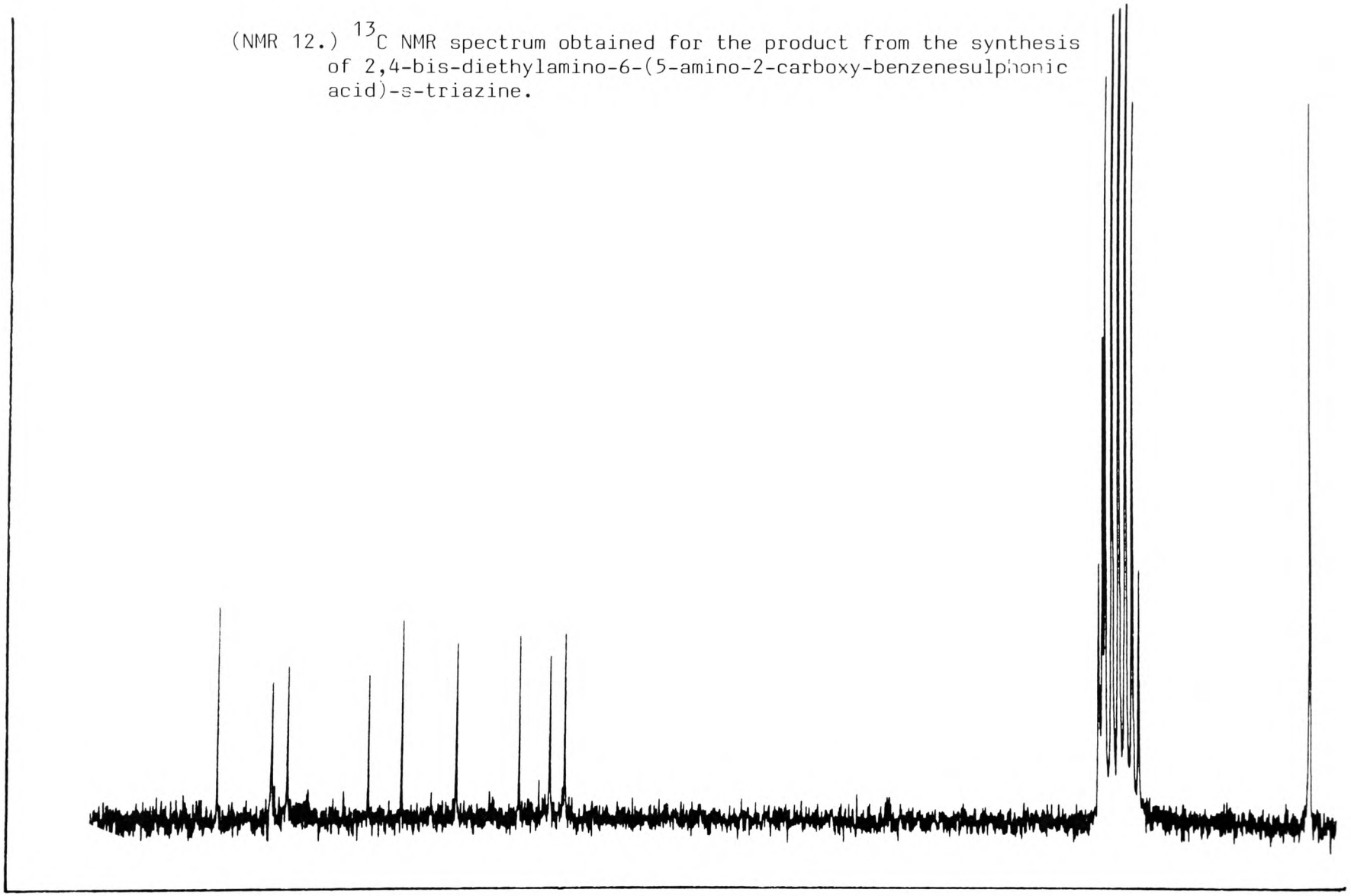
(NMR 10.) ^{13}C NMR spectrum obtained for the product from the synthesis of 5-Amino-2-carboxy-benzenesulphonic acid.



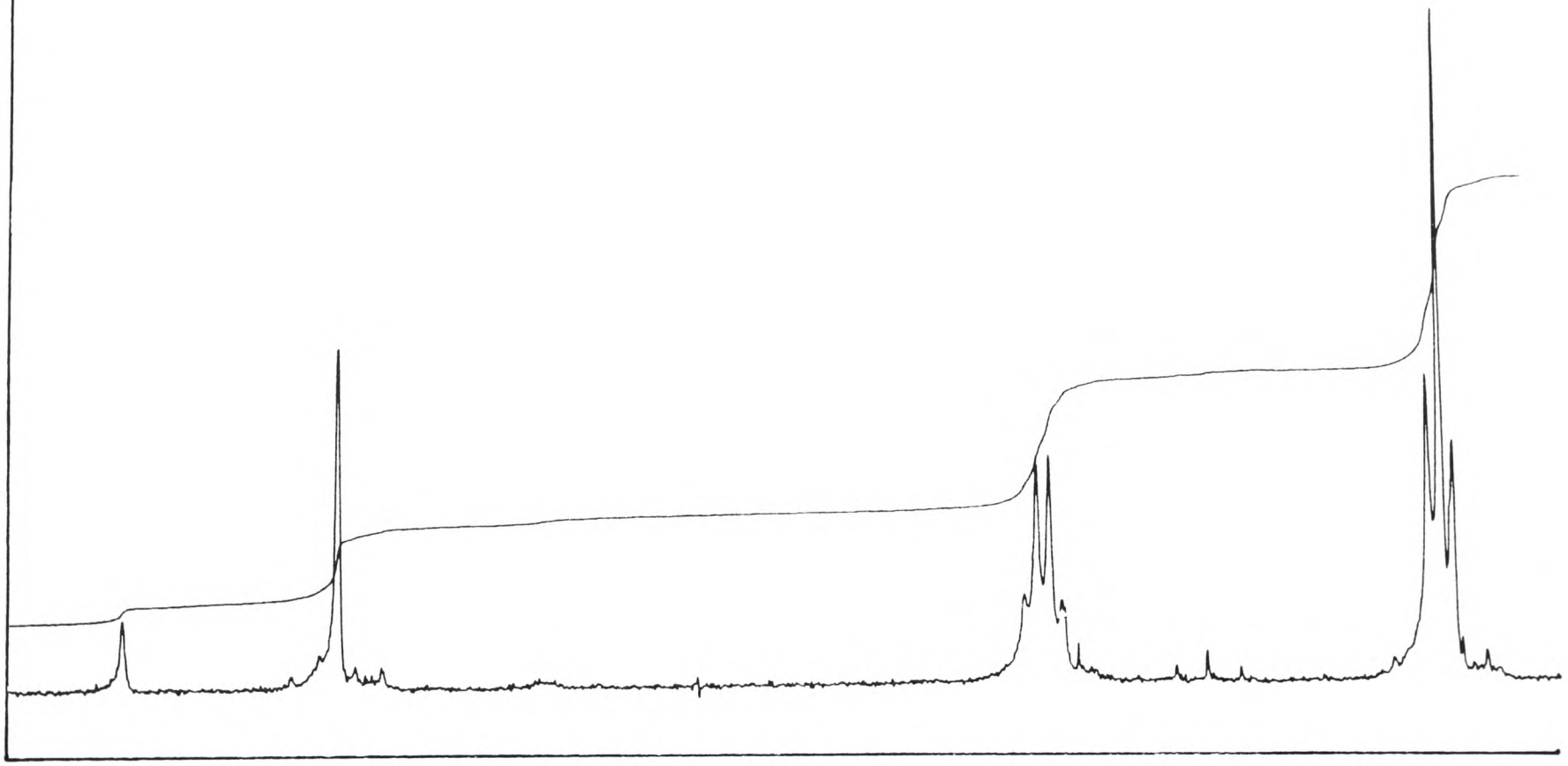
(NMR 11.) ^{1}H NMR spectrum obtained for the product from the synthesis of 2,4-bis-diethylamino-6-(5-amino-2-carboxy-benzenesulphonic acid)-s-triazine.



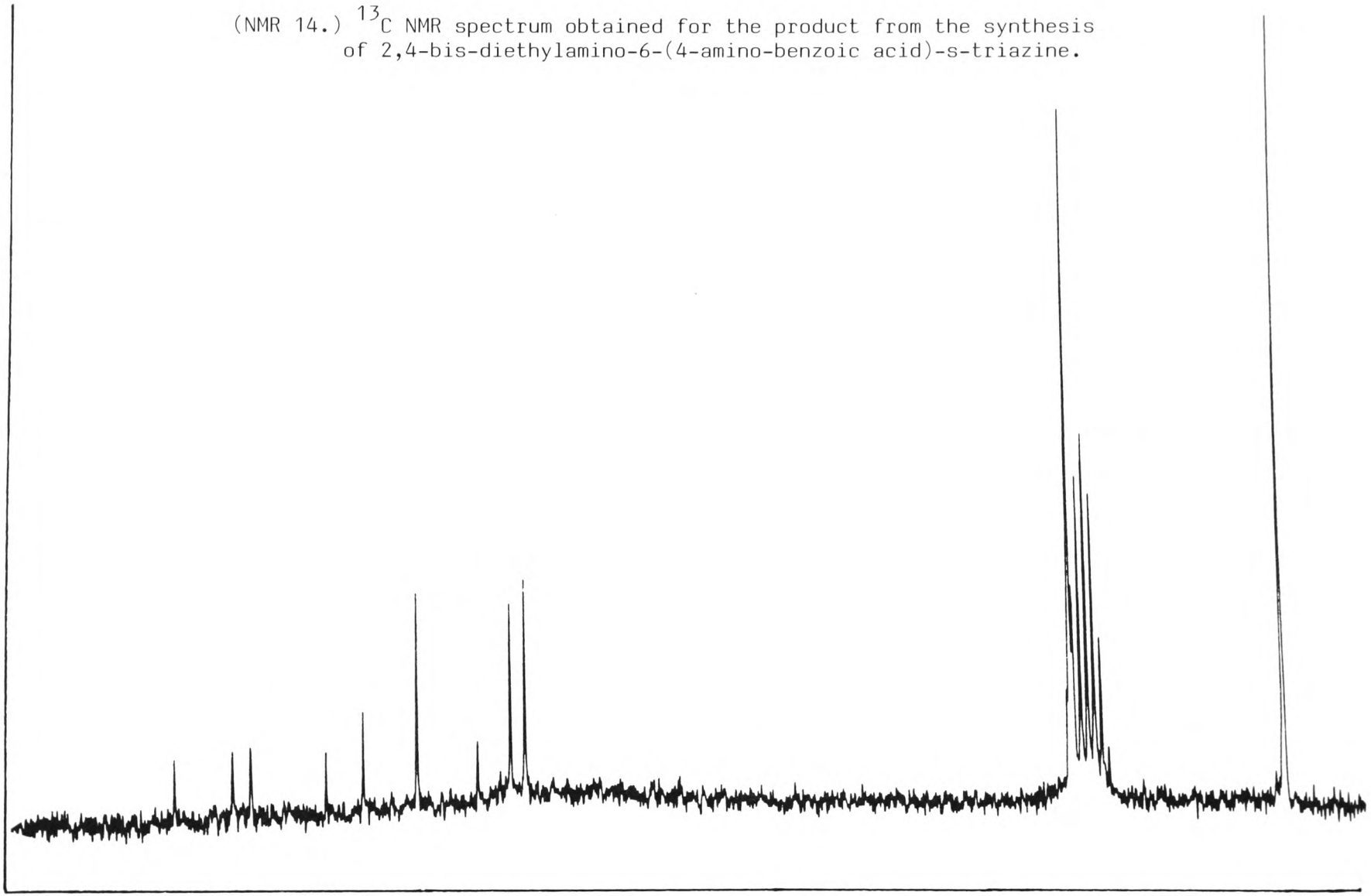
(NMR 12.) ^{13}C NMR spectrum obtained for the product from the synthesis of 2,4-bis-diethylamino-6-(5-amino-2-carboxy-benzenesulphonic acid)-s-triazine.



(NMR 13.) ^{1}H NMR spectrum obtained for the product from the synthesis of 2,4-bis-diethylamino-6-(4-amino-benzoic acid)-s-triazine.



(NMR 14.) ^{13}C NMR spectrum obtained for the product from the synthesis of 2,4-bis-diethylamino-6-(4-amino-benzoic acid)-s-triazine.



APPENDIX 5.

MASS SPECTRA.

The mass spectra were obtained by The Thames Water Authority.

Introduction.

As with IR and NMR it is not intended to give a definitive explanation of mass spectroscopy but only a brief explanation prior to interpreting some spectra.[67, 68, 69]

In principle, the function of the mass spectrometer is relatively simple. The molecules of the substance, in a gaseous phase, become ionised, the ions produced are accelerated in an electric field of high potential, they become deviated in a magnetic field and then arrive at a collector, generating a signal the intensity of which is proportional to the number of ions arriving. The whole apparatus operates under high vacuum.

The principle types of ions that appear in a mass spectrum include: molecular ions, isotopic ions, fragment ions, rearrangement ions, multiply charged ions, metastable ions, negative ions and ions by ion-molecular interaction.

The ion formed by the loss of one electron from part of the molecule is called the 'molecular ion' or 'parent ion'. The molecular ion is the precursor of all other ions in the spectrum and requires the minimum energy for its formation. The nominal mass of such an ion corresponds to the molecular weight of the compound. The relative intensity of the molecular ion depends on its stability with respect to the decomposition products, and it thus indicates the type of compound under investigation.

The molecular ion contains the most abundant isotopes of the constituent elements. The parent peak is always accompanied by other peaks of higher mass that are caused by the ions which contain the heavier isotopes.

Fragment ions are formed by the fragmentation of the molecular ion in

the ion source. Because of the considerable energy of the electronic bombardment usually employed to ionise an organic substance, the molecular ion, being in an excited state, tends to break some of its internuclear bonds, producing fragments of lower mass called "ionic fragments". The study of the fragmentation patterns of the most abundant ions can furnish structural information on the compound being analysed. The mass spectrum of a molecule consists of numerous peaks, some of which are intense, whereas others are weak or scarcely visible. The preferential formation of some ions depends on the molecules tendency to break some bonds rather than others and on the stability of some fragments because of their particular structure.

To obtain the molecular formula of a compound, it is necessary to measure the accurate mass of the molecular ion and hence high resolution instruments must be used. Knowing the accurate mass, the molecular formulae can be obtained from tables or by calculation.

Although much information can be obtained from the examination of the mass spectrum of a compound, it can be difficult and often impossible to identify all the peaks present. In this investigation we are looking for further confirmation of the compounds synthesised, for which purpose we need only examine the first few fragmentation ions.

Analysis of the spectra obtained during the synthesis of the model FWA.

(a) 2,4-bis-(diethylamino)-6-chloro-s-triazine:

Examination of the mass spectrum (MS 1) obtained for the product from the synthesis of the above compound yields the following information:

Molecular weight ----- 257

Peak at $^{m}/e$ 259 approximately one third the intensity of the molecular peak indicating the presence of one chlorine atom in the molecule.

Peak at $^{m}/e$ 242 ----- loss of CH_3 from the molecule.

Peak at $^{m}/e$ 228 ----- loss of CH_3CH_2 from the molecule.

All the above are consistent with the spectrum expected for 2,4-bis-(diethylamino)-6-chloro-s-triazine.

(b) 5-Amino-2-carboxy-benzenesulphonic acid:

Examination of the mass spectrum (MS 2) obtained for the product from the synthesis of the above compound yields the following information:

Molecular weight ----- 199 This is not in agreement with the molecular weight of the above compound but is in agreement with that of the anhydride.

Peak at $^{m}/e$ 199 ----- due to loss of SO_3 from the molecule.

Peak at $^{m}/e$ 119 ----- due to loss of CO from the molecule.

All the above are consistent with the spectrum expected for the anhydride of 5-amino-2-carboxy-benzenesulphonic acid.

(c) 2,4-bis-(diethylamino)-6-(5-amin0-2-carboxy-benzenesulphonic acid)-s-triazine.

Examination of the mass spectrum (MS 3) obtained for the product from the synthesis of the above compound yields the following information:

Molecular weight ----- 420 This is in agreement with the molecular weight of the anhydride of the above compound.

Peak at $^{m}/e$ 405 ----- loss of CH_3 from the molecule.

Peak at $^{m}/e$ 391 ----- loss of CH_3CH2 from the molecule.

All the above are consistent with the spectrum expected for the anhydride of the above compound.

(d) 2,4-Bis-(diethylamino)-6-(4-amino-benzoic acid)-s-triazine.

Examination of the spectrum (MS 4) obtained for the product from the synthesis of the above compound yields the following information:

Molecular weight ----- 358

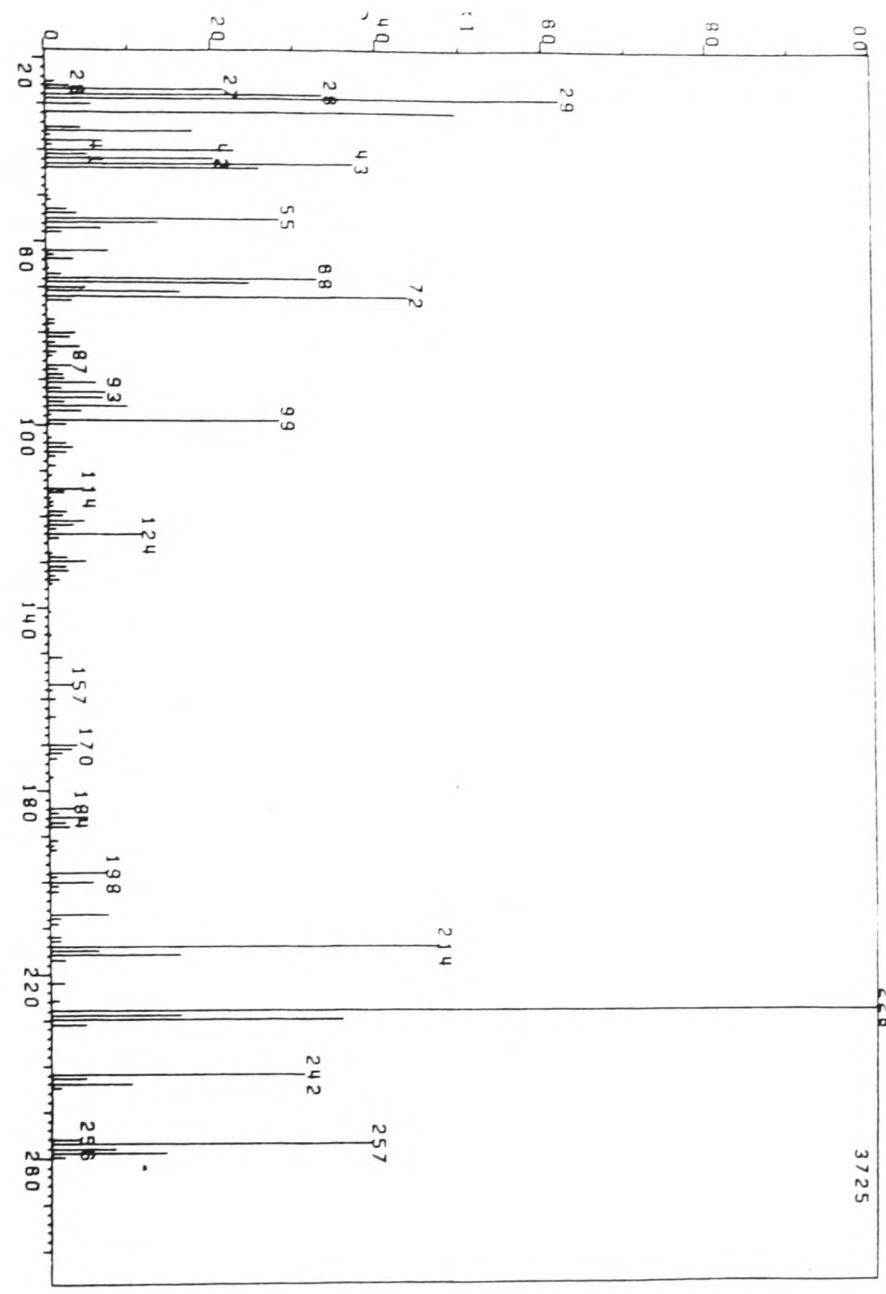
Peak at $^{m}/e$ 343 ----- loss of CH_3 from the molecule.

Peak at $^{m}/e$ 329 ----- loss of CH_3CH_2 from the molecule.

Peak at $^{m}/e$ 315 ----- loss of N from the ion at 329.

All the above are consistent with the spectrum expected for 2,4-bis (diethylamino)-6-(4-amino-benzoic acid)-s-triazine.

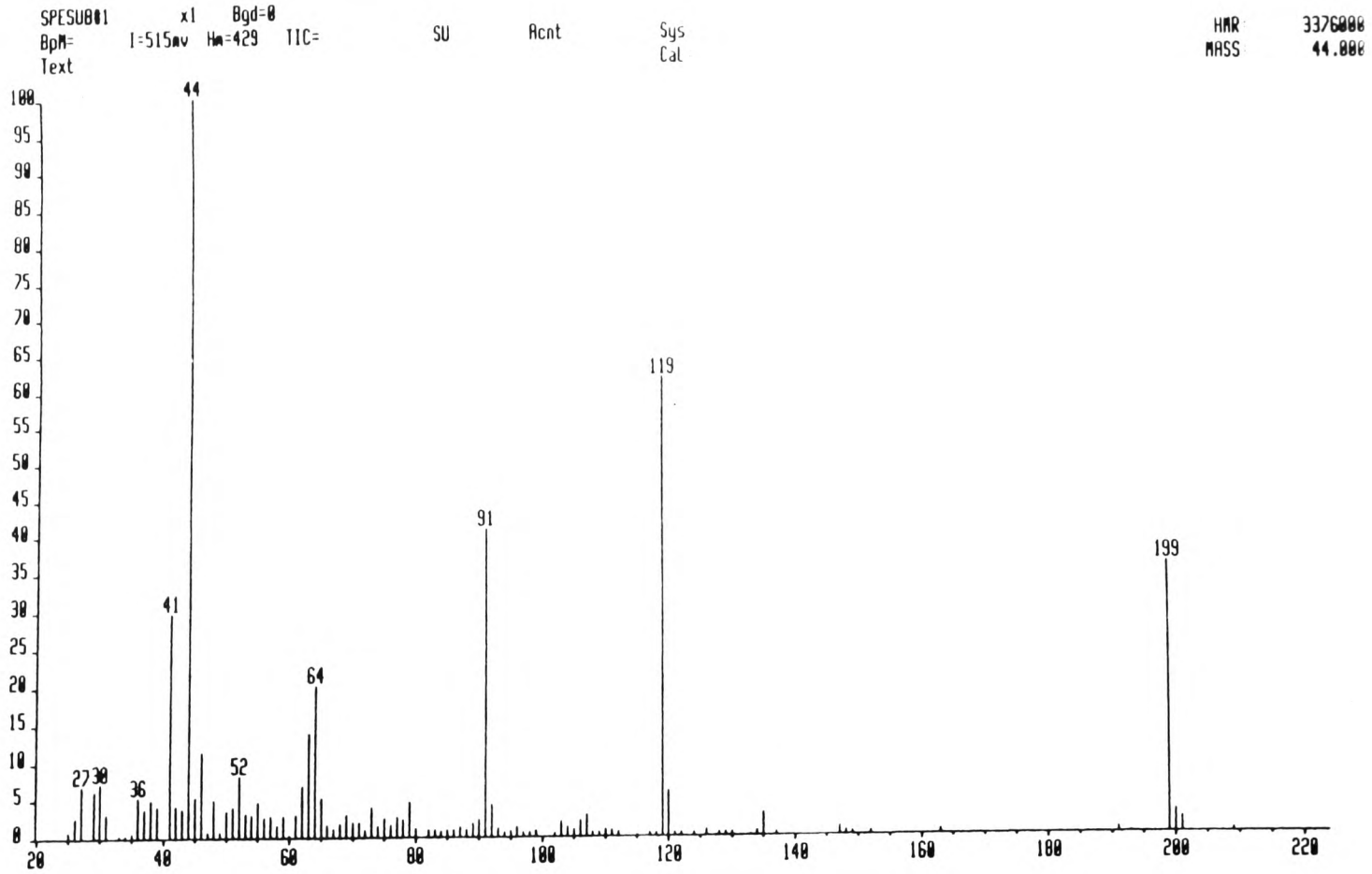
(MS 1.) Mass spectrum obtained for the product from the synthesis of 2,4-bis-diethylamino-6-chloro-s-triazine.



EM100.0 RT 0:49:50 16-FEB-84 TIC 306 AUTO GAIN:1 1. 1
BASE INT.:3725 B/G SCAN:2113 STATUS:5E
• 2,4-BIS(DIETHYLAMINO)-6-CHLORO-S-TRIAZINE

PEAK NO.	MASS	%AR. MOD.	%AR. BASE	ABS AREA
1	25.3630	1.21	1.21	45
2	26.3281	2.95	2.95	110
3	27.2583	21.23	21.23	791
4	28.1348	33.40	33.40	1244
5	29.0137	61.91	61.91	2306
6	29.0486	5.56	5.56	207
7	32.0190	49.32	49.32	1837
8	33.1103	0.13	0.13	5
9	35.1514	4.24	4.24	158
10	36.1523	17.64	17.64	657
11	37.1094	0.70	0.70	26
12	38.0566	6.85	6.85	255
13	39.0234	0.81	0.81	30
14	39.8560	22.58	22.58	841
15	41.0132	5.02	5.02	187
16	42.0972	20.21	20.21	753
17	43.1567	37.05	37.05	1380
18	44.1601	25.66	25.66	956
19	49.0063	0.30	0.30	11
20	49.9536	0.38	0.38	14
21	51.0229	0.72	0.72	27
22	52.1045	0.08	0.08	3
23	53.1567	2.60	2.60	97
24	54.1821	3.65	3.65	136
25	55.1929	28.08	28.08	1046
26	56.1890	13.37	13.37	498
27	57.2021	6.63	6.63	247
28	58.1421	1.88	1.88	70
29	62.0703	7.38	7.38	275
30	63.1470	0.91	0.91	34
31	64.1260	3.30	3.30	123
32	65.2026	0.19	0.19	7
33	66.1816	0.30	0.30	11
34	67.1924	1.80	1.80	67
35	68.1226	32.59	32.59	1214
36	69.1016	24.38	24.38	908
37	70.0732	4.70	4.70	175
38	71.1206	15.97	15.97	595
39	72.1850	43.52	43.52	1621
40	73.2129	3.11	3.11	116
41	75.1953	0.16	0.16	6
42	77.1753	0.91	0.91	34
43	78.1494	0.99	0.99	37
44	79.1016	0.40	0.40	15
45	80.0415	3.36	3.36	125
46	81.1157	2.68	2.68	100
47	82.1680	0.91	0.91	34
48	83.1982	3.84	3.84	143
49	84.2309	1.21	1.21	45
50	85.2417	0.67	0.67	25
51	87.0720	2.95	2.95	110
52	88.0615	1.26	1.26	47
53	89.0080	1.99	1.99	74
54	89.9780	2.17	2.17	81
55	91.0571	5.80	5.80	216
56	92.0423	1.74	1.74	65
57	93.1055	6.95	6.95	259
58	94.1406	6.60	6.60	246
59	95.1758	2.07	2.07	77
60	96.1401	9.56	9.56	356
61	97.1509	4.05	4.05	151
62	98.1641	0.43	0.43	16
63	99.1040	27.89	27.89	1039
64	100.0756	2.28	2.28	85
65	103.0907	0.54	0.54	20
66	104.1015	2.23	2.23	83
67	105.1415	3.01	3.01	112
68	106.1547	2.34	2.34	87
69	107.1166	0.91	0.91	34
70	109.1161	0.97	0.97	36
71	111.1376	0.51	0.51	19
72	113.6449	3.92	3.92	146
73	114.6703	1.88	1.88	70
74	116.1229	0.40	0.40	15
75	117.1509	0.70	0.70	26
76	118.1176	0.72	0.72	27
77	119.1161	2.28	2.28	85
78	120.1220	1.77	1.77	66
79	121.1327	4.38	4.38	163
80	122.1777	3.06	3.06	114
81	123.2202	1.05	1.05	39
82	124.2016	10.93	10.93	407
83	125.2123	1.29	1.29	48
84	128.0981	0.51	0.51	19
85	129.0478	2.20	2.20	82
86	130.0317	4.56	4.56	170
87	131.0595	2.04	2.04	76
88	132.0849	2.47	2.47	92
89	133.1102	0.86	0.86	32
90	134.1699	1.37	1.37	51
91	135.1635	0.51	0.51	19
92	141.1010	0.40	0.40	15
93	146.0497	0.40	0.40	15
94	151.1718	1.56	1.56	58
95	157.0824	2.66	2.66	99
96	158.0663	0.46	0.46	17
97	160.0390	0.83	0.83	31
98	164.1527	0.83	0.83	31
99	169.9975	3.46	3.46	129
100	171.0668	2.77	2.77	103
101	172.0921	1.69	1.69	63
102	173.1176	0.94	0.94	35
103	177.0507	0.43	0.43	16
104	184.0732	2.68	2.68	100
105	185.1122	1.15	1.15	43
106	186.1083	4.59	4.59	171
107	187.1484	1.88	1.88	70
108	188.1444	2.39	2.39	89
109	191.0424	1.02	1.02	38
110	192.0849	0.43	0.43	16
111	193.0786	0.89	0.89	33
112	198.0565	6.42	6.42	239
113	199.0503	0.81	0.81	30
114	200.0488	5.18	5.18	193
115	201.0498	0.94	0.94	35
116	202.0995	0.94	0.94	35
117	207.0482	7.01	7.01	261
118	208.0492	1.29	1.29	48
119	209.0477	0.99	0.99	37
120	212.0922	1.34	1.34	50
121	213.1396	1.23	1.23	46
122	214.0820	46.26	46.26	1723
123	215.1293	5.77	5.77	215
124	216.1254	15.46	15.46	576
125	217.1703	1.85	1.85	69
126	222.1728	1.66	1.66	62
127	226.0863	0.99	0.99	37
128	228.0420	100.00	100.00	3725
129	229.0209	15.49	15.49	577
130	229.9975	34.98	34.98	1303
131	231.0277	4.19	4.19	156
132	242.0336	30.34	30.34	1130
133	243.1126	4.24	4.24	158
134	244.0698	9.53	9.53	355
135	245.1488	1.21	1.21	45
136	256.1107	3.28	3.28	122
137	257.0971	38.28	38.28	1426
138	258.0858	7.60	7.60	283
139	259.0721	13.77	13.77	513
140	260.1268	1.69	1.69	63

(MS 2.) Mass spectrum obtained for the product from the synthesis of 5-Amino-2-carboxy-benzesulphonic acid.

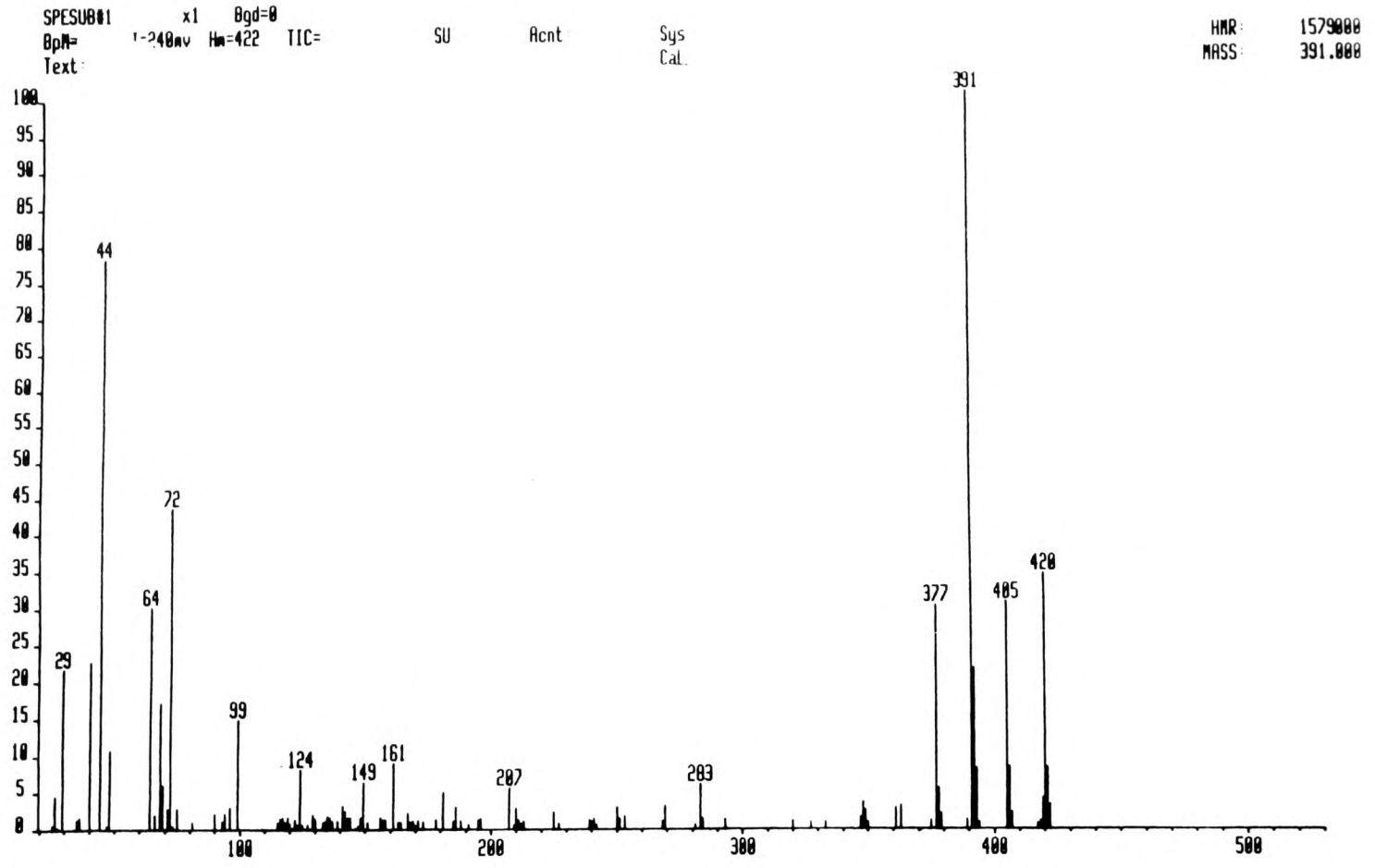


Vs Hm:429 TIC:0 SU Acnt: Sys: Cal:

Text:5-Amino-2-carboxy-benzenesulphonic acid

Peak No	Mass	% Base
1	199	35.6
2	193	0.0
3	191	0.5
4	163	0.4
5	152	0.3
6	149	0.3
7	148	0.5
8	147	1.0
9	137	0.4
10	135	2.8
11	134	0.4
12	133	0.0
13	130	0.4
14	129	0.4
15	128	0.4
16	126	0.7
17	124	0.3
18	122	0.4
19	121	0.3
20	120	6.0
21	119	61.3
22	118	0.4
23	117	0.3
24	115	0.1
25	112	0.5
26	111	0.7
27	110	0.8
28	109	0.4
29	108	0.5
30	107	2.7
31	106	2.0
32	105	0.9
33	104	1.2
34	103	1.8
35	102	0.3
36	99	0.6
37	98	0.4
38	97	0.5
39	96	1.2
40	95	0.7
41	94	0.5
42	93	1.1
43	92	4.3
44	91	40.8
45	90	2.2
46	89	1.6
47	88	0.9
48	87	1.2
49	86	0.8
50	85	0.8
51	84	0.6
52	83	0.8
53	82	0.8
54	80	0.9
55	79	4.5
56	78	2.2
57	77	2.5
58	76	1.5
59	75	2.4
60	74	1.3
61	73	1.9
62	72	0.8
63	71	1.8
64	70	1.9
65	69	2.8
66	68	1.7
67	67	0.9
68	66	1.6
69	65	5.1
70	64	20.0
71	63	13.6
72	62	6.7
73	61	2.9
74	60	0.1
75	59	2.8
76	58	1.5
77	57	2.6
78	56	2.6
79	55	4.5
80	54	2.8
81	53	3.0
82	52	0.8
83	51	3.9
84	50	3.3
85	49	0.7
86	48	4.8
87	47	0.7
88	46	11.3
89	45	5.2
90	44	100.0
91	43	3.7
92	42	4.1
93	41	29.7
94	39	4.1
95	38	4.8
96	37	3.7
97	36	5.3
98	35	0.6
99	34	0.2
100	33	0.1
101	31	1.0
102	30	2.0
103	29	0
104	27	6.7
105	26	2.5
106	25	0.6

(MS 3.) Mass spectrum obtained for the product from the synthesis of 2,4-bis-diethylamino-6-(5-amino-2-carboxy-benzenesulphonic acid)-s-triazine.

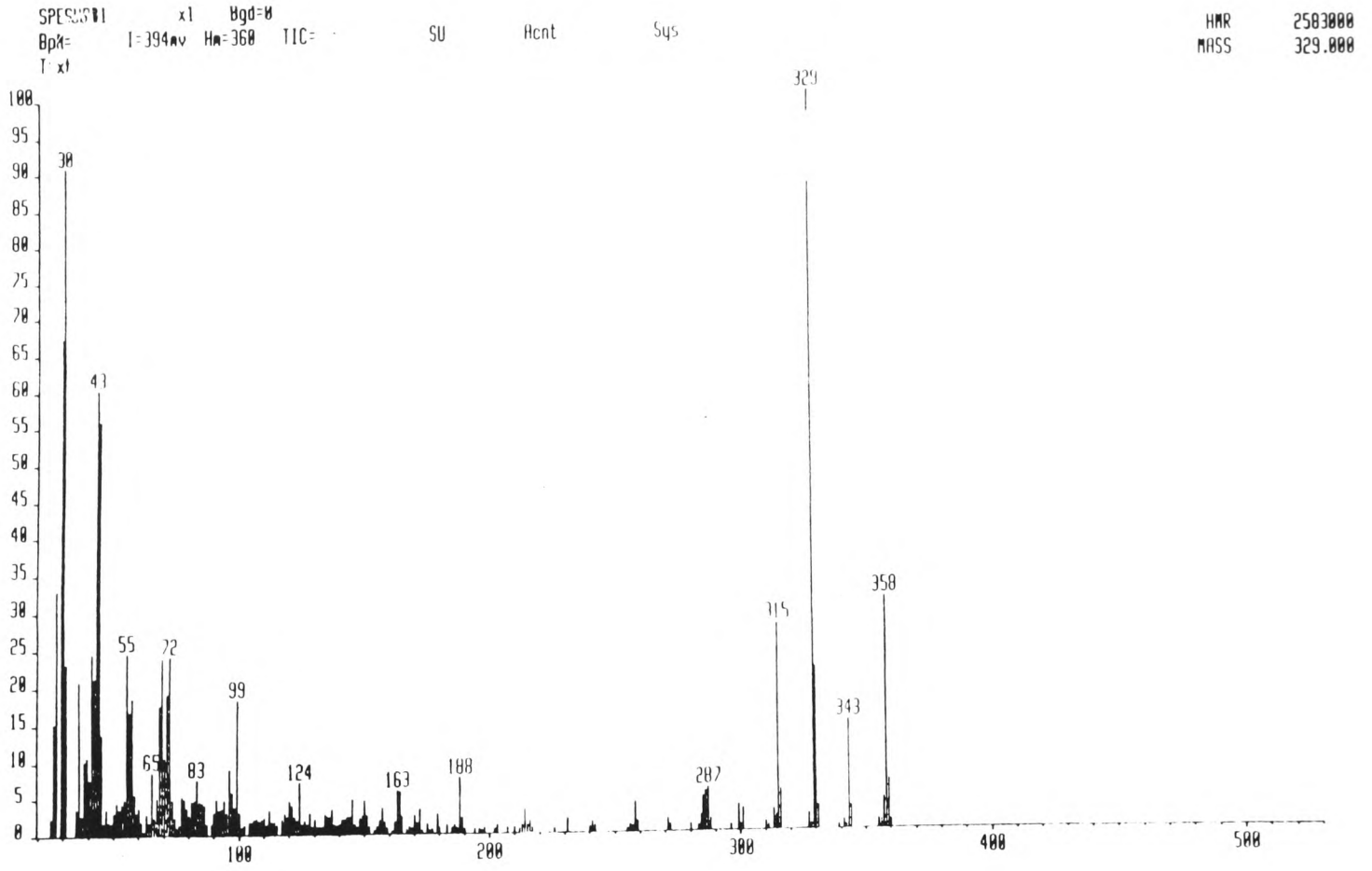


CS HM 422 TIC:0

Text: 2,4-bis-(diethylamino)-6-(5-amino-2-carboxy-benzenesulphonic acid)-S-triazine

Peak No	Mass	% Base	Mass	% Base	Mass	% Base
1	422	3.2	63	64	163	0.0
2	421	0.3	64	65	161	0.7
3	420	34.2	65	66	158	1.1
4	419	4.1	66	67	157	1.1
5	418	1.1	67	68	156	1.6
6	417	0.7	68	69	151	0.0
7	407	2.2	69	70	149	6.2
8	406	8.2	70	71	148	1.5
9	405	30.3	71	72	147	0.3
10	394	0.9	72	73	146	0.1
11	393	0.0	73	74	144	1.6
12	392	21.5	74	75	143	1.5
13	391	100.0	75	76	142	2.3
14	389	1.2	76	77	141	1.1
15	379	2.0	77	78	139	1.1
16	378	5.3	78	79	137	1.0
17	377	30.0	79	80	136	1.5
18	375	1.0	80	81	135	1.7
19	362	3.0	81	82	134	1.0
20	361	2.7	82	83	131	0.9
21	350	0.0	83	84	130	1.4
22	349	2.6	84	85	129	1.5
23	348	3.6	85	86	127	0.5
24	347	1.6	86	87	125	0.6
25	333	0.9	87	88	124	7.9
26	327	0.8	88	89	123	0.4
27	320	1.0	89	90	122	1.1
28	293	1.2	90	91	121	0.0
29	284	1.3	91	92	120	0.7
30	283	5.8	92	93	119	0.0
31	281	0.9	93	94	118	1.5
32	269	3.1	94	95	117	0.0
33	260	1.0	95	96	116	1.1
34	253	1.6	96	97	115	0.0
35	251	1.3	97	98	109	0.0
36	250	2.0	98	99	107	14.7
37	242	0.6	99	100	96	2.0
38	241	1.3	100	101	94	2.0
39	240	1.1	101	102	93	0.5
40	239	1.2	102	103	92	2.0
41	227	0.9	103	104	91	0.0
42	225	2.2	104	105	75	2.7
43	213	1.0	105	106	73	6.3
44	212	0.7	106	107	72	4.4
45	211	1.2	107	108	71	2.7
46	210	2.7	108	109	69	5.9
47	209	0.6	109	110	68	17.0
48	207	5.4	110	111	66	1.0
49	196	1.3	111	112	64	30.0
50	195	1.2	112	113	48	10.6
51	191	0.6	113	114	47	0.4
52	188	1.1	114	115	44	78.2
53	186	2.8	115	116	40	22.5
54	185	1.1	116	117	36	1.5
55	181	4.9	117	118	35	1.2
56	178	1.2	118	119	29	21.6
57	173	0.0	119	120	27	0.7
58	171	1.0	120	121	26	4.3
59	170	0.3	121	122	25	0.5
60	169	0.9	122			
61	168	0.0				
62	167	2.0				

(MS 4.) Mass spectrum obtained for the product from the synthesis of 2,4-bis-diethylamino-6-(4-amino_benzoic acid)-s-triazine.



vs Hm:360 TIC:0 SU Acnt:
Text:

Peak No	Mass	% Base	Peak No	Mass	base	Peak No	Mass	% Base
1	360	1.1	51	212	0.6	101	141	1.0
2	359	6.5	52	210	0.7	102	140	1.1
3	358	30.8	53	207	0.6	103	139	1.0
4	357	4.1	54	203	1.0	104	138	1.2
5	356	1.1	55	202	0.6	105	137	3.1
6	355	1.1	56	198	0.6	106	136	2.2
7	344	3.0	57	197	0.4	107	135	2.2
8	343	14.4	58	196	0.5	108	134	2.5
9	342	0.5	59	194	0.5	109	133	0.8
10	341	1.2	60	190	0.5	110	132	0.8
11	339	0.5	61	189	2.0	111	131	0.9
12	331	3.0	62	188	7.4	112	130	1.0
13	330	21.5	63	187	0.6	113	129	0.9
14	329	100.0	64	186	1.0	114	128	2.7
15	328	0.7	65	185	0.6	115	127	1.2
16	327	2.1	66	183	1.1	116	126	1.7
17	316	5.3	67	180	0.7	117	125	1.1
18	315	27.4	68	179	2.6	118	124	6.9
19	314	1.8	69	177	0.4	119	123	1.7
20	313	2.7	70	176	0.3	120	122	2.2
21	311	0.5	71	175	1.1	121	121	3.8
22	310	1.1	72	173	0.4	122	120	4.4
23	301	2.9	73	172	3.2	123	119	2.1
24	300	0.7	74	171	1.4	124	118	2.7
25	299	3.4	75	170	2.3	125	117	1.9
26	296	0.7	76	169	0.7	126	115	1.2
27	288	1.5	77	168	0.9	127	114	1.5
28	287	5.7	78	167	0.4	128	113	1.5
29	286	5.3	79	165	2.1	129	112	3.2
30	285	4.6	80	164	5.6	130	111	1.2
31	284	2.1	81	163	5.7	131	110	2.2
32	283	0.6	82	162	1.6	132	109	1.9
33	280	0.9	83	161	0.4	133	108	1.7
34	272	0.8	84	159	0.7	134	107	2.1
35	271	1.7	85	158	1.5	135	106	1.9
36	259	1.4	86	157	3.4	136	105	1.5
37	258	3.8	87	156	1.7	137	104	1.5
38	257	0.7	88	155	0.8	138	102	1.2
39	256	0.8	89	154	0.4	139	101	0.8
40	255	0.4	90	152	0.9	140	100	2.8
41	242	0.9	91	151	2.3	141	99	10.0
42	241	1.3	92	150	4.4	142	98	3.6
43	240	0.7	93	149	2.1	143	97	5.6
44	231	1.8	94	148	2.1	144	96	0.7
45	226	0.4	95	147	0.9	145	95	2.8
46	217	0.6	96	146	1.2	146	94	4.5
47	216	1.6	97	145	4.6	147	93	3.3
48	215	1.1	98	144	2.2	148	92	3.3
49	214	3.3	99	143	2.1	149	91	4.7
50	213	1.0	100	142	1.9	150	90	2.9

Scan 360 (0.000 min): 5U.D
Base Peak: 360 (1.00 Million)
TIC: 0

Acnt:

Sys:

Text: 2,4-Bis-(diethylamino)-6-(4-amino-carboxylic acid)-S-triazine

Peak No	Mass	% Base	Peak No	Mass	% Base
151	89	1.4	201	38	10.0
152	87	1.3	202	37	2.6
153	86	3.9	203	36	20.7
154	85	4.2	204	35	3.4
155	84	4.4	205	31	23.1
156	83	7.4	206	30	90.7
157	82	4.5	207	29	67.2
158	81	4.4	208	27	32.9
159	80	2.6	209	26	15.0
160	79	3.6	210	25	2.2
161	78	4.7			
162	77	5.0			
163	76	1.2			
164	75	0.9			
165	74	2.2			
166	73	4.5			
167	72	24.0			
168	71	18.9			
169	70	10.3			
170	69	23.7			
171	68	17.3			
172	67	5.0			
173	66	2.1			
174	65	0.5			
175	64	1.5			
176	63	2.6			
177	62	0.3			
178	61	1.7			
179	60	3.5			
180	59	2.9			
181	58	5.4			
182	57	18.4			
183	56	16.5			
184	55	24.5			
185	54	4.7			
186	53	4.1			
187	52	3.3			
188	51	4.3			
189	50	2.9			
190	49	1.4			
191	48	1.7			
192	47	3.3			
193	46	1.5			
194	45	13.4			
195	44	55.7			
196	43	60.1			
197	42	21.1			
198	41	24.5			
199	40	7.4			
200	39	10.5			

APPENDIX 6.

H. P. L. C. DETAILS.

Introduction.

Chromatography is a general term applied to a wide variety of separation techniques based upon the sample partitioning between a moving phase, which can be a gas or liquid, and a stationary phase, which may be either a liquid or a solid.

High performance liquid chromatography (HPLC) (or "high pressure" or "high speed" liquid chromatography) was developed from liquid chromatography (LC) which was performed in large diameter columns under essentially atmospheric conditions; analysis times were long and the entire procedure was generally very tedious.

Kirkland and Huber developed high pressure systems in 1968 operating at pressures up to 3000 p.s.i. In this method small diameter columns (1-3mm) with support particle sizes in the region of 30μm are used and the mobile phase is pumped through the column at a high flow rate (ca 1 to 5 $cm^3\ min^{-1}$). Separations by this method may be effected much more rapidly (about 100 times faster) than by the use of conventional liquid chromatography.

In the early stages of LC the stationary phase (polar or non polar) was coated onto an inert support and packed into a column. The mobile phase was then passed through the column. To meet the need for more robust columns, packing with the stationary phase chemically bonded to the inert support were developed. This form of partition chromatography is called bonded-phase chromatography (BPC) and has rapidly become one of the most popular forms of HPLC. HPLC is termed "normal phase" if the stationary phase is more polar than the mobile phase and "reverse phase" if the mobile phase is more polar than the stationary phase.

It is not intended here to give exact details of the mechanism of

separation of sample components - there are many good textbooks available on the subject - but to give an "overall view" of how separations can be made and improved.

The column is the heart of the chromatogram. The success or failure of an analysis depends on the choice of column and proper operating conditions.

Columns are almost invariably made from stainless steel. The columns are generally operated at ambient temperatures, but elevated temperatures have been used, particularly in ion-exchange and exclusion chromatography.

The packings are dependent on the type of compounds to be separated: - eg their molecular weights; if they are water soluble or not; if they are ionic/non-ionic etc.: - most HPLC textbooks give a rough guide to packing selection.

In liquid chromatography the composition of the solvent or mobile phase is one of the variables influencing the separation. There is a wide variety of solvents used in all the modes of HPLC, but there are several desirable properties which are common to all of them. The mobile phase should:

(a) Be pure; no contaminants,

(b) Not react with the packing,

(c) Be compatible with the detector,

(d) Dissolve the sample,

(e) Have a low viscosity,

(f) Permit easy sample recovery, if desired,

(g) Be commercially available at a reasonable price.

In general the solvent is discarded after use since cleanup procedures are tedious and expensive. Of all the requirements above, the first four

are the most important.

Quite often a mixture of solvents, rather than a single solvent, is used as the mobile phase; this can enhance and speed up separation. Another advantage of using mixed solvents is that a technique know as gradient elution can be used.

Gradient elution is a technique whereby the ratio of the solvents are varied as the analysis is carried out. In the case of a simple two solvent system the composition of the mobile phase can be changed from 100% A to 100% B and most modern instruments allow very complex gradient elutions to be carried out. By the use of gradient elution, total analysis time can be significantly reduced, resolution and peak shape can be greatly enhanced. The optimum gradient is found by trial and error.

A detector is required to sense the presence and measure the amount of a sample component in the column effluent. Good detectors exhibit high sensitivity, low noise, a wide linear response range, and response to all types of compounds. A low sensitivity to flow and temperature fluctuations is desirable, but not always obtainable.

Details of the HPLC systems used in the biodegradation studies.

Solvent: - 50% methanol/50% water

Column packing: - Zorbax TMS

Flow rate 2µl min^{-1} at 210 ats. max ($25^{o}C$)

Detector 254nm UV

HPLC Instrument: - Varian with data station.

Loop injection

HPLC Separation of the Two Model Compounds

Same conditions as in the biodegradation studies except for the solvents and percentages used.

1. Water/methanol; 100% Water/0% Methanol to 0% Water/100% Methanol.
2. Water/acetonitrile as in 1.
3. methanol/ acetonitrile as in 1.

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